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STUDIES ON THE MIGRATION AND TRANSFORMATION OF LYMPHOCYTES IN IMMUNIZED AND NON-IMMUNIZED ANIMALS

BY

HANS DIDERHOLM

EJNAR MUNKSGAARD
COPENHAGEN
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Translated by

Marianne Nordfors, M.A.

Almqvist & Wiksells

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CHAPTER I

Introduction

Production of lymphocytes

Lymphocytes are mainly produced in the lymph nodes, the lymphoid tissue of the alimentary canal, the thymus, spleen and possibly also bone marrow. According to *Yoffey & Courtice* (1956), the mesenteric lymph nodes and the lymphoid tissue of the intestine are probably the main source of blood lymphocytes. The authors base this assumption on investigations into the number of lymphocytes in the intestinal and thoracic duct lymph (*Yoffey* 1933 and 1936, *Sanders, Florey & Barnes* 1940 and *Mann & Higgins* 1950).

The production of lymphocytes in the thymus has been investigated by means of mitotic counts (*Kindred* 1942, *Andreasen & Christensen* 1949, *Leblond* 1959, *Ito* 1959 and others) and by measurements of the turnover of deoxyribonucleic acid (DNA) with the aid of radioactive isotopes (*Andreasen & Ottesen* 1944 and 1945, *Lundin* 1958, *Cooper* 1958 and others). Most of this research has shown the thymus to be an important centre for lymphocyte formation. Opinions differ, however, concerning the extent to which lymphocytes produced in the thymus degenerate there and to what proportion they reach the blood. Calculations based on *Kindred's* values indicate that about 90% of the small lymphocytes enter the blood. The thymic contribution to maintaining the lymphocyte level in the blood has also been studied by means of thymectomy (*Schooley & Kelly* 1958 and *Bierring* 1960). In these investigations a distinct decrease in the output of lymphocytes from the thoracic duct as well as in the number of lymphocytes in the blood was noted after thymectomy. This may indicate that the thymus is an important source of blood lymphocytes, but there are also other interpretations of the results.

The investigations by *Kindred* (1942) and *Andreasen & Christensen* (1949) into the frequency of mitotic figures in lymphatic and lymphoid tissues show that the spleen, too, produces lymphocytes

but to a lesser extent than the thymus and lymph nodes. This is in good agreement with *Andreasen & Ottesen's* (1944 and 1945) studies on the production of lymphocytes with the aid of radioactive isotopes. It is, however, not at all clear how great a proportion of the lymphocytes produced in the spleen is released to the blood (*Yoffey & Courtice* 1956).

The bone marrow contains a large number of lymphocytes. A point of argument has been whether these are myelopetal or myelofugal, i.e. whether they pass to the marrow from the blood, or in the reverse direction. Since lymphocytes in the bone marrow form perivascular aggregations rather than follicles and mitoses are rare in these, this organ is not considered an important producer of lymphocytes (*Yoffey & Courtice* 1956).

No accurate information exists concerning the total rate of lymphocyte production in the body. *Kindred* (1942), calculating from mitotic counts and assuming a 30 minute cycle of mitosis, estimated about 16 million cells per hour in rats weighing 200 g. *Ito* (1959), using the colchicine technique on rats of the same weight, arrived at about the same figure.

It is well known that there are many factors, hormonal, nutritional, antigenic and others, that influence lymphopoiesis. Experiments where antigens were administered suggested that these had a stimulating effect (*Awaya* 1956, *Gyllensten, Ringertz & Ringertz* 1956, *Fichtelius, Gisslén & Hassler* 1957, *Lundin* 1958 and others). This is also supported by the fact that in guinea-pigs raised in a bacteria-free environment the lymphatic and lymphoid tissues are not fully developed (*Glimstedt* 1936).

Release of lymphocytes from the producer organs to the blood and length of their stay in the blood

Large numbers of lymphocytes are daily transported from the producer organs to the blood via the thoracic duct. *Yoffey* (1936) has called the ratio between the number of lymphocytes daily released to the blood via this duct and the number of lymphocytes in the blood the Daily Replacement Factor (DRF). The latter has been calculated for some of the most common experimental animals. Thus the DRF of the rat is about 10 (*Gowans* 1957 and *Keohane & Metcalf* 1958), of the rabbit about 3 (*Sanders et al.* 1940) and of the guinea-pig about 4 (*Yoffey* 1956). It must, however, be

pointed out that there are many sources of blood lymphocytes besides the thoracic duct. Probably the output is quite considerable via the right lymph duct and other channels, and in addition some of the cells are presumably released directly to the blood within the lymphocyte-producing organs (*Yoffey & Courtice* 1956).

A high DRF strongly indicates that the average time spent by the lymphocytes in the blood is short. Investigations using the isotope technique have suggested that the mean life-span of lymphocytes is probably several weeks (*Osgood, Tivey, Davison, Seaman & Li* 1952, *Ottesen* 1954, *Hamilton* 1956, 1957 and 1959, *Resegotti* 1957). The period spent in the blood is presumably a very small part of the life-span of the cells.

Fate of lymphocytes after leaving the blood

If the number of lymphocytes in the blood is constant and if no lymphocytes are destroyed there, as many cells must leave the blood during a certain period as are released to it. It is not quite known how great a proportion of lymphocytes is actually destroyed in the blood. According to *Bunting & Huston* (1921) and *Wiseman* (1932), it is a relatively small part. The number of lymphocytes daily released from the blood is probably very large. The fate of these cells has been the subject of much research and discussion. Many possibilities have been suggested:

(1) The cells may recirculate, i.e. they return to the blood via the lymph and the lymph nodes (*Sjövall* 1936). *Yoffey & Drinker* (1939) have investigated the number of cells in the peripheral and central lymph and come to the conclusion that only a small part of the cells recirculates. They were, however, criticized by *Farr* (1951) and *Fichtelius* (1953), who pointed out that the lymphocytes might take the route blood–lymph node lymph, thus avoiding the peripheral lymph altogether. *Gowan's* (1957, 1959 *a* and *b*) investigations, in which the thoracic duct was drained after transfusion of lymphocytes, argue a recirculation of greater proportions than that suggested by *Yoffey & Drinker* (1939).

(2) The cells may migrate to the mucous membranes of the digestive and respiratory tracts whence they are excreted into the lumen. The problem has been studied and discussed by amongst others *Bunting & Huston* (1921), *Stenquist* (1934), *Andrew & Sosa*

(1947), *Andrew & Burns* (1947) and *Ambrus & Ambrus* (1959). Opinions differ concerning the magnitude of the supposed excretion of lymphocytes. Probably rather few or hardly any at all pass from the blood through the epithelium of the digestive and respiratory tracts.

(3) The cells may migrate to the skin. A large number of lymphocytes is found in the epidermis. The fate of these is as yet unclear. *Andrew & Andrew* (1949) maintain that there is nothing to indicate that the lymphocytes degenerate within the epidermis, and they believe that these cells can turn into ordinary epithelial cells. Against this there is the assertion of *Andreasen* (1952 and 1959) that the lymphocytes do degenerate within the epidermis. The problem of the origin of lymphocytes in the skin has been studied by means of transfusion experiments with labelled lymphocytes (references in Chapter VII) and also by means of thymectomy experiments (*Fichtelius, Danielsson & Hallander* 1960). The investigations have shown that thymus lymphocytes probably migrate to the skin.

(4) The cells may enter the bone marrow, where they can function as progenitors of other types of blood cells, both red and white. *Farr* (1951) has transfused lymphocytes labelled with a fluorescent dye and recovered some of these in the bone marrow amongst other organs. He found that some of the transfused lymphocytes had been transformed into myelocytes. This assertion was criticized by *Keohane & Metcalf* (1958), who pointed out that these myelocytes might simply be cells that had taken up fluorescent dye released from the transfused cells. *Yoffey & Courtice* (1956) give a survey of the subject.

(5) The cells may migrate to the liver and spleen. This assumption is based mainly on transfusion experiments using different techniques (references in Chapter VII). *Fichtelius* (1953, 1957 *c* and 1960) has put forward the hypothesis that in the process of antibody formation thymus lymphocytes migrate to the spleen, where they are transformed into plasma cells. To support his hypothesis he refers to certain investigations showing that there are hardly any antibodies in the thymus after immunization (*Bjørnebo, Gormsen & Lundquist* 1947 and *Harris, Rhoads & Stokes* 1947), but that thymectomy causes a reduction of the antibody content in serum (*Hammar* 1938, *Inzerillo & Chiti* 1953 and *Fichtelius, Laurell & Philipsson* 1961).

(6) The cells may pass into the connective tissue all over the body. According to *Trowell* (1958), a large proportion of the lymphocytes in the body are probably connective tissue lymphocytes. The fate of these is not wholly known but at least some of them find their way into lymphatic capillaries (*Yoffey & Drinker* 1939).

Functions of lymphocytes

The problems of the functions of lymphocytes are intimately connected with the fate of these cells after leaving the blood. A few functions have already been suggested in connection with this question, but it must be pointed out that there is no direct evidence for any of these or for most of the other functions attributed to lymphocytes. In a general paper on lymphocytes *Trowell* (1958) says: "Although lymphocytes have been studied for over a hundred years, not a single function can yet be ascribed to them with any confidence, and their role in the body remains both an enigma and a challenge. Broadly speaking, two rival and mutually exclusive views have been entertained. The one is that lymphocytes are primitive stem cells capable of differentiating into all the other types of blood cells, and also into reticulo-endothelial cells and fibroblasts. The other is that they are end cells dying after a few hours without having performed any known function." Lymphocytes have also been credited with the ability to turn into epithelioid cells, giant cells, epidermal cells and plasma cells. General accounts concerning the supposed function of lymphocytes as stem cells of other cells are given by *Fichtelius* (1953), *Sundberg* (1955), *Yoffey & Courtice* (1956), *Trowell* (1958) and others. Much of the evidence for lymphocyte transformation is based upon tissue culture experiments, which may be criticized on certain points (*Fichtelius* 1953). *Downey* (1917), however, has shown that lymphocytes can turn into macrophages within the vessels. *Farr's* (1951) observations concerning a transformation of lymphocytes into myelocytes have already been discussed. By means of transfusion experiments *Fichtelius* (1957 *b*) has shown that thymus lymphocytes labelled with P^{32} can be transformed into exudate cells, presumably mainly macrophages. More recently investigations have been made concerning the transformation of lymphocytes into plasma cells, and these are discussed in Chapter IV.

To the lymphocytes has been ascribed the function of synthesizing, storing and transporting nucleoproteins for use by other cells. In support of their ability to act as trephocytes certain characteristics of these cells have been adduced, such as their size, motility, ubiquitous distribution and rapid turnover. The subject has been comprehensively discussed by *Kelsall & Crabb* (1958).

Lymphocytes play a part in the body's defence against infections, but how they function in this connection is not yet known. *Bunting & Huston* (1921) and *Sjövall* (1936) are of the opinion that lymphocytes can take up toxins. The ability of these cells to differentiate into macrophages, plasma cells and other cells which characterize the histology of inflammation has already been discussed. The question what cells produce antibodies has long been a matter of dispute. It seems that the plasma cells are mainly responsible for forming antibodies, but probably lymphocytes, too, play an important part, not least in certain types of hypersensitivity. The comprehensive literature on the subject has been reviewed by *Fagraeus* 1948, *Wesslén* 1952, *Yoffey & Courtice* 1956, *Trowell* 1958, *Burnet* 1959 and others.

Difference between thymus lymphocytes and other lymphocytes

In what respects thymus lymphocytes differ from others has not yet been discovered. Morphologically they seem much alike, but the fact that the thymus sometimes reacts differently from lymphatic tissue suggests that they may differ for other reasons. As it is unknown what proportion of the lymphocytes in the blood comes from the thymus and since we are unable to differentiate such lymphocytes from others, it is impossible to calculate the time spent by the thymus lymphocytes in the blood. As to the fate of these cells after leaving the blood, certain differences exist between thymus and lymph node lymphocytes according to transfusion experiments with labelled cells (*Fichtelius* 1958 *a* and *b*, *Diderholm & Fichtelius* 1959 *b*). Since the rôle of lymphocytes in the body is still a matter of conjecture, it is difficult to express an opinion about the extent to which the function of thymus lymphocytes differs from that of other lymphocytes.

Purpose and plan of the investigations

The present studies seek to clarify the problem to what organs and what regions of these organs the lymphocytes, especially thymus lymphocytes, migrate and also deal with the ability of these cells to differentiate into other types of cells in immunized and non-immunized animals. The project includes three types of experiments:

(1) Transfusion experiments with labelled thymus lymphocytes are carried out. The cells are labelled *in vitro*, in some cases with H^3-thymidine, in others with S^{35}-methionine, and autologous transfusions are made on guinea-pigs. The animals are killed at different intervals after transfusion and certain organs are examined by means of stripping film autoradiography. The autoradiograms are compared in order to find out whether the H^3- and S^{35}-labelled lymphocytes are distributed differently after transfusion. The presence of labelled cells of types other than those transfused strongly indicates that a transformation of the transfused cells has taken place. Some of the animals are immunized before transfusion to stimulate the formation of plasma cells.

(2) Immunized and non-immunized guinea-pigs are used to study the influence of subtotal thymectomy on the weights of spleen and lymph nodes, on the volumes of red and white splenic pulps and on the plasma cell content of the spleen. If the thymus lymphocytes migrate to the organs in question under physiological conditions and if no compensatory mechanism is developed after the ectomy, the weights of these organs should be lower in the experimental animals than in sham-operated controls. If one of the splenic pulps is more dependent on thymus lymphocytes than the other, the volume of the first pulp should differ for ectomized and sham-operated animals. Moreover, according to the hypothesis concerning the function of thymus lymphocytes in antibody formation, one would expect the plasma cell content of the spleen to be lower in ectomized than in sham-operated animals.

(3) The number and distribution of labelled cells in the spleen and thymus of rats are studied by means of stripping film autoradiography at different intervals after the administration of H^3-thymidine, which is incorporated into the DNA of the cells. The distribution of labelled cells a short time after thymidine has been administered yields information concerning the regions within the

organ where the synthesis of DNA is taking place. At a later point labelled cells may to a relatively large extent consist of cells that have migrated to the organ in question. In order to find support for the assumption that lymphocytes migrate to the spleen, a comparison is made between autoradiograms from various intervals after thymidine has been injected.

CHAPTER II

The Fate in Guinea-Pigs of Transfused Thymus Lymphocytes Labelled *in vitro* with H^3-Thymidine

In order to study the fate of the lymphocytes after leaving the blood, transfusion experiments have been carried out with labelled as well as unlabelled lymphocytes. *Osogoe* (1950), working with rabbits, made homologous transfusions of unlabelled lymphocytes from the lymph nodes. Forty-eight to seventy-two hours after the transfusion accumulations of lymphocytes were observed in the interlobular spaces of the liver and in the perifollicular regions of the spleen. *Osogoe & Hitachi* (1950) made a similar experiment with thymus lymphocytes. In this case, too, accumulations of lymphocytes were found in the above-mentioned parts. In both investigations it was emphasized that these accumulations consisted of the transfused cells, since none were obtained if the cells were killed before the transfusion.

Autologous transfusions of lymph node lymphocytes labelled with a fluorescent dye were carried out on rabbits by *Farr* (1951). He recovered the cells after 2–3 and 12 hours in the bone marrow, thymus, mesenteric lymph node, Peyer's patches and in the white pulp of the spleen. After 12 hours most of the labelled cells in the bone marrow were myelocytes, which, according to the author, had been formed by a transformation of the transfused lymphocytes. As previously mentioned, this assertion was criticized by *Keohane & Metcalf* (1958). These authors have also used fluorescent dye when labelling lymphocytes for transfusion. For 2 hours after autologous, homologous and heterologous transfusions of thymus lymphocytes to rats the labelling was sufficiently strong for them to observe the cells in the lung, kidney, liver, spleen and thoracic duct. No differences were observed between the autologous and homologous cells, and the heterologous ones, taken from guinea-pigs. *Hagerman* (1954) used guinea-pigs sensitized with dinitrochlorobenzene in making homologous transfusions of thymus

lymphocytes labelled with a fluorescent dye. The author thinks it very probable that the labelled cells he found in the skin after 1 and 2 hours were the transfused ones.

Of recent years a common method of labelling lymphocytes for transfusion has been to use radioactive isotopes. *Weisberger, Guyton, Heinle & Storaasli* (1951) used rats for homologous transfusions of P^{32}-labelled lymphocytes taken from an intestinal lymphatic vessel. After 10 minutes the cells were recovered in the lung and spleen, and possibly also in the kidney and liver. *Fichtelius* (1953), too, used P^{32} for labelling lymphocytes. He made homologous as well as heterologous transfusions to rats, which were killed after 24 hours. The author showed that homologous thymus lymphocytes were recovered in the spleen and liver, while heterologous thymus lymphocytes (from rabbits) could be traced to the liver only. Later *Fichtelius* (1958 a) transfused lymph node lymphocytes labelled with P^{32} to rats. After 24 hours he recovered the homologous cells in the liver and bone marrow, and probably also in the lymph nodes and lung, while the heterologous cells (from rabbits) were found only in the liver.

If the lymphocytes are labelled with a radioactive isotope and the autoradiographic method is used, the transfused cells can be located within the organs of the recipient. With the aid of apposition autoradiography *Diderholm & Fichtelius* (1959 a) traced transfused thymus lymphocytes labelled with P^{32} to the red pulp of the spleen and the non-follicular regions of the hepatic lymph node. In a later work (*Diderholm & Fichtelius* 1959 b) lymph node lymphocytes were traced to the non-follicular regions of the mesenteric lymph node as well as to the regions mentioned above. These experiments consisted of homologous transfusions to guinea-pigs, which were killed after 48 hours. Further transfusion investigations with lymphocytes are reported in Chapter VII.

The above-mentioned autoradiographic investigations have certain weaknesses. The resolving power of the autoradiograms was so low that the transfused cells could not be distinguished individually and it was thus impossible to study a possible transformation of the lymphocytes into other types of cells. It was, moreover, necessary to transfuse large quantities of lymphocytes. If stripping film autoradiography is used and the cells are labelled with an isotope emitting low-energy beta particles, these weaknesses can be eliminated. In the present work thymus lymphocytes

are labelled with H^3 and stripping film autoradiography is used. The labelling has been carried out *in vitro*, thus making autologous transfusions possible.

Material and methods

Animals

24 male guinea-pigs, about 4 weeks old and weighing 141–224 g, were used. They were of the same strain and were given a standardized diet.

Excision of the Thymus

The animal was placed on its back and after infiltration anaesthesia with 0.5% Xylocain[1] an incision about 15 mm long was made in the median line at the level of the mandibular angles. The two large symmetrical thymus lobes situated directly under the platysma were excised, while the minor aberrant thymus lobes were left *in situ*. 2 or 3 cutaneous sutures were made. The whole operation took 2–3 minutes.

Preparation of the Cell Suspension

After fat and adjacent tissue had been removed, the thymus was placed in a siliconed glass beaker and cut into small pieces after which 1 cc Parker's solution was added. After the thymus had been cut still finer, filtration through gauze took place. The resulting filtrate was composed of a cell suspension with a negligible quantity of small threads of connective tissue.

Labelling the Cells

In connection with filtration the cell suspension was transferred to a 30 cc siliconed glass beaker (diameter 30 mm), where more Parker's solution was added, making the volume 0.9 cc. To the suspension was added 0.4 μC H^3-thymidine,[2] which had a specific activity of 360 mC per mmole. The thymidine had been dissolved in physiological saline, and 0.05 cc was added to each suspension.

The glass beaker containing the cell suspension was corked and kept in a water-bath at 37°C for 60 minutes.

In 10 suspensions to be injected into control animals the cells were

[1] Supplied by Astra AB, Sweden.
[2] Supplied by Schwartz Laboratories Inc., New York.

killed by being kept in an oven at 53–55°C for 30 minutes after first having been labelled.

In order to investigate the approximate frequency of labelled cells homologous serum was added to a small part of each suspension, after which smears were made and subsequently autoradiographed. The results are given below.

Control of the Viability of the Cells

In order to investigate the presence of dead cells in the cell suspensions, *Schrek's* (1943) test was used. To 0.02 cc suspension was added 0.38 cc Tyrode's solution, in which eosin had been dissolved at a concentration of 1 : 2000. The cells stained red were considered to be dead, and their frequency was about 2–8%. This frequency was more or less the same in the few cases where dead cells were counted also before labelling. In the suspensions heated after labelling practically all the cells were stained.

Transfusion

No attempt was made to wash the cells, in order to avoid injuring them. 0.6 cc of the suspension was injected into the deep jugular vein, a siliconed syringe being used. The number of cells injected into the experimental animals was on the average ca 9.8×10^7 (ca $3.1 \times 10^7 - 13.6 \times 10^7$) and into the control animals ca 10.6×10^7 (ca $3.5 \times 10^7 - 20.5 \times 10^7$). After the transfusion, which took 3–4 minutes, the wound was once more sutured. Sterile precautions were maintained during the entire course of the investigation.

Altogether 24 transfusions were carried out, of which 10 on control animals. Each of the latter received cell suspension heated after labelling plus 0.4 μC H^3-thymidine dissolved in 0.05 cc physiological saline. In all other respects these animals and their organs were treated in exactly the same way as the others. Two of the animals that had received living cells were rather dull for 2–3 hours after the transfusion, while all the others were quite unaffected by it.

In order to avoid possible differences due to diurnal changes, the transfusions were carried out about the same time of day.

Killing the Animals

The animals were killed 8 hours after the transfusion. The wound was cut open under ether anaesthesia and about 0.5 cc blood taken from the deep jugular vein to be used for investigating the presence of labelled cells. Immediately afterwards the animals were killed by a blow on the neck.

Histological Technique

The spleen, the large mesenteric lymph node situated at the main stems of the mesenteric vessels, the distal part of the ileum and pieces

of the right hepatic lobe, right lung and skin from the abdomen were dissected out. Blood and bone marrow smears were made. Bone marrow was taken from the right femur. Cell suspensions were prepared of pieces of spleen and lymph node and smeared out. All smears were made on glass slides that had been treated with a solution of 5 g gelatin and 0.5 g chrome alum in 1000 cc distilled water. This was done in order to prevent the autoradiographic film from coming off the glass. The smears were fixed after air-drying in absolute methyl alcohol. After fixation the smears of the thymus cell suspensions were transferred to distilled water in order to wash away surplus H^3-thymidine as well as any other substances containing H^3 not incorporated in DNA (*Pelc & La Cour* 1959 and *Crathorn & Shooter* 1960). They were kept for two hours in the water, which was changed several times.

The pieces of organ were fixed in neutral 10% formalin and embedded in paraffin. Sections 6 μ thick were cut. In order to ensure that no labelled cell was registered more than once, only every 4th or 5th section was used. They were stretched on glass slides that had also been treated with the solution of gelatin and chrome alum in distilled water. Before the film was applied, deparaffinization took place. The staining technique is described below.

Autoradiographic Technique

Kodak autoradiographic stripping plates (AR 10) were used. The stripping procedure was carried out largely according to the directions of *Doniach & Pelc* (1950). After being covered with the film, the slides were dried in air in front of a fan and were then stored at about 4°C in light-proof boxes containing silica gel. The exposure time was 35 days. The films were then developed in Kodak D 19 b for 5 minutes at 18–20°C and rinsed in water for 30 seconds. Thereafter they were fixed in Kodak F 52 for 7 minutes and rinsed in running water for 20 minutes.

As a rule about 20 sections of each organ were autoradiographed. An exception was the spleen, of which at least 50 sections were examined. From every animal about 3 smears each of blood, bone marrow and the prepared suspensions were autoradiographed.

Staining

All stains of sections as well as smears were carried out after development. Some of the sections were stained with Ehrlich's haematoxylin, others with methyl green-pyronin according to Unna-Pappenheim. The smears were stained with Giemsa.

Examination of the Autoradiograms

Before a cell was considered to be labelled, at least 3 grains were required over it. This applied to both smears and sections. The number,

Table 1. *Percentage of H^3-labelled lymphocytes in the spleen observed in the red pulp, mean grain count over the labelled lymphocytes and mean number of these cells per 100 fields of vision in the spleen where 12 determinations were made.*

Determination no.	Percentage of labelled lymphocytes in red pulp	Mean grain count over labelled lymphocytes	Mean number of labelled lymphocytes per 100 fields of vision
1	88.5	13.1	101.3
2	91.0	13.8	108.9
3	90.1	14.3	104.0
4	89.7	14.3	101.1
5	91.8	14.1	106.0
6	90.8	14.5	99.2
7	90.8	13.8	100.9
8	89.0	14.2	101.8
9	93.0	14.7	104.3
10	90.2	14.3	104.7
11	93.0	14.5	101.0
12	91.7	14.0	104.1
Mean and standard deviation	90.8±1.4	14.1±0.4	103.1±2.7
Coefficient of variation	±1.6	±3.0	±2.6

position and morphology of labelled cells were examined in the organs preserved. The spleens were more thoroughly examined than the other organs, 1000 labelled lymphocytes being counted in each. The location of these, i.e. their presence in the red or white pulp, was examined. The perifollicular zone described by *Hartmann* (1930), *Krumbhaar* (1948) and others was considered to be part of the white pulp. The number of grains over every 3rd cell of these 1000 cells was also counted. Moreover the mean number of labelled lymphocytes per 100 fields of vision was determined (a field of vision corresponded to an area of about 7.8×10^3 μ^2 of the section).

In order to get an idea of the random error when 1000 lymphocytes are counted, 12 such determinations were made from one and the same spleen. The results are shown in Table 1.

Results

The thymus cell suspensions, which consisted of ca 95–98% lymphocytes, contained about 7–12% labelled cells. Practically all

TABLE 2. *Percentage of H^3-labelled lymphocytes in the spleen observed in the red pulp, mean grain count over the labelled lymphocytes and mean number of these cells per 100 fields of vision in the spleen.*

Animal no.	Percentage of labelled lymphocytes in red pulp	Mean grain count over labelled lymphocytes	Mean number of labelled lymphocytes per 100 fields of vision
1	90.8	14.1	103.1
2	92.0	14.6	32.7
3	89.9	12.2	38.4
4	92.2	14.8	70.0
5	90.2	13.1	45.2
6	86.7	12.7	14.6
7	92.7	15.6	40.5
8	92.3	13.6	38.6
9	93.9	14.7	74.9
10	92.0	13.2	36.1
11	91.3	14.8	44.9
12	91.1	13.9	32.4
13	89.0	12.1	15.6
14	89.4	13.6	26.2
Mean and standard error	91.0±0.5	13.8±0.3	43.8±6.4
Coefficient of variation	±2.0	±7.6	±54.8

of these were lymphocytes, but a few reticular cells and some unidentifiable cells were also observed. Of the labelled lymphocytes the majority were of medium size, the rest being either large or small. Medium-sized lymphocytes were those with a diameter of 7–11 μ. Lymphocytes with a diameter greater than 11 μ and less than 7 μ were classified as large and small respectively. About 60–85% of all the lymphocytes belonged to the small class. Plate 1 shows labelled lymphocytes from a thymus cell suspension.

In all animals labelled lymphocytes were found in the spleen. The number of these varied considerably from one animal to the other. Most of the lymphocytes were found in the red pulp, as shown in Table 2, and their frequency per field of vision was greater here than in the white pulp. Sometimes the cells were concentrated to regions near the white pulp. In the latter pulp no difference was observed between the frequencies of labelled lympho-

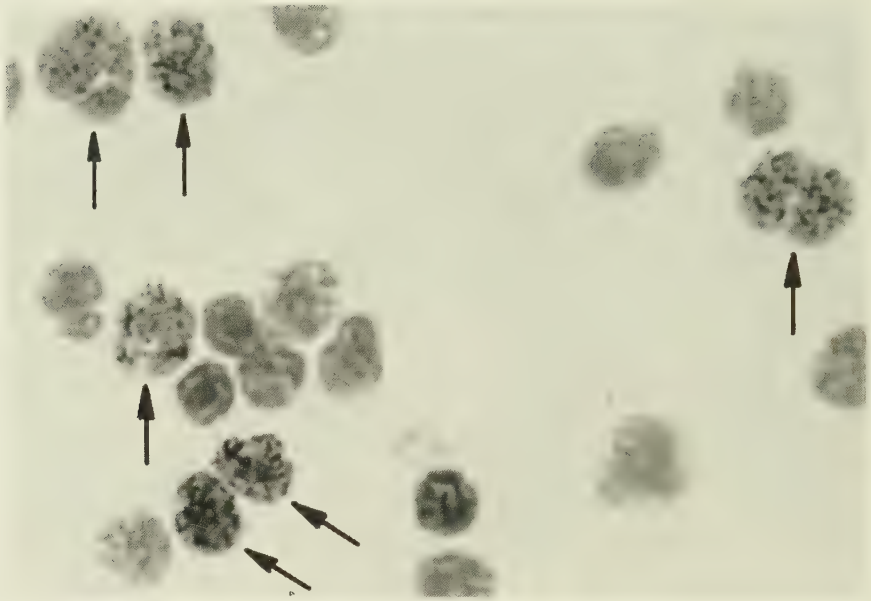

Plate 1. Autoradiogram of a smear from a thymus cell suspension labelled *in vitro* with H^3-thymidine. Six lymphocytes are labelled. Only a few background grains are seen.
(Giemsa, × 1400.)

Plate 2. Autoradiogram of a section from a spleen showing two H^3-labelled lymphocytes in the red pulp. Eight hours after the autologous transfusion.
(Haematoxylin, × 850.)

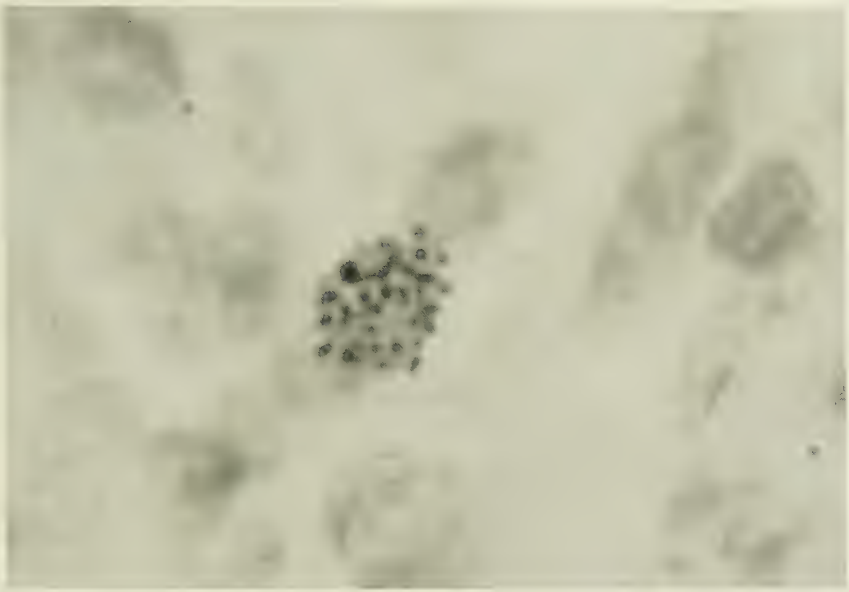

Plate 3. Highly magnified H^3-labelled lymphocyte in the red pulp of the same spleen as in Plate 2. Note the high resolving power of the autoradiogram; almost all grains are localized over the cell.
(Haematoxylin, × 2200.)

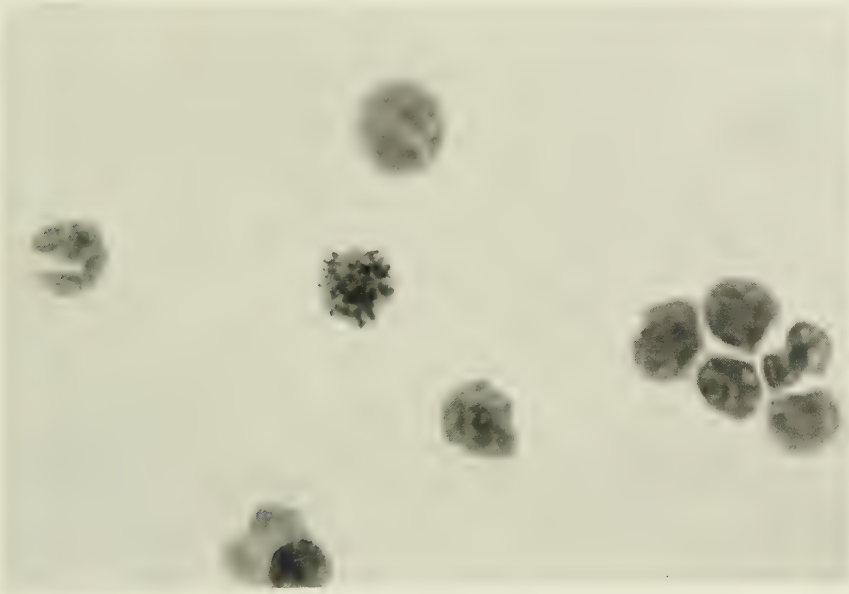

Plate 4. Autoradiogram of a bone marrow smear showing one H^3-labelled lymphocyte. Eight hours after the autologous transfusion. Only a few background grains are seen.
(Giemsa, × 1400.)

cytes per field of vision in the perifollicular zones, the germinal centres and the other parts. Table 2 also shows the mean numbers of grains counted over the labelled lymphocytes and the mean numbers of labelled lymphocytes per 100 fields of vision. As is seen from the Table, a mean number of 91.0% of the lymphocytes was found in the red pulp and the variations between the animals were rather small. For the individual animals the mean grain count over the labelled lymphocytes varied betwen 12.1 and 15.6, the mean for the whole material being 13.8. The mean number of labelled lymphocytes per 100 fields of vision was for the individuals 14.6–103.1, for the whole material 43.8.

A few labelled reticular cells and some labelled cells that could not be identified were also observed in the spleen. No labelled plasma cells were present, however. No difference between the distribution of labelled lymphocytes and other labelled cells could be noticed. Plates 2 and 3 show labelled lymphocytes in the spleen.

Labelled lymphocytes were also found in the liver of all the animals. The frequency of these varied considerably. In all cases, however, the number of lymphocytes per 100 fields of vision was much smaller than in the spleen of the same animal, about 1/10–1/20. Most of the cells were diffusely spread over the lobules, while a few were found in the interlobular connective tissue. Most of the labelled cells in the liver were classified as lymphocytes. The remaining labelled cells could not be identified. No difference was observed concerning the location of the lymphocytes and the other labelled cells.

As to the remaining organs that were cut into sections, stray labelled lymphocytes were discovered in a few animals in the mesenteric lymph node and the lung only. In the former organ they were diffusely spread over both medulla and cortex with the exception of the follicles, in which only stray ones were found. In the lung the labelled lympocytes were recovered in the alveolar walls. In both organs some other labelled cells were also present, but these could not be identified. However, their location appeared to agree with that of the labelled lymphocytes. In all the organs cut into sections the labelled cells practically always occurred singly or, exceptionally in twos or threes.

Stray labelled cells were found in the bone marrow smears of a few animals only. Almost all these cells were lymphocytes, the exceptions being some reticular cells and a few others that could

not be identified. Plate 4 shows an autoradiogram of a bone marrow smear.

A comparison between the degrees of labelling of the lymphocytes in the various organs revealed no certain differences according to the autoradiograms of the sections. The same can on the whole be said of the autoradiograms of the smears. However, occasionally some of the labelled lymphocytes in the thymus cell suspensions were rather more strongly labelled than those in the other smears.

The autoradiograms of the wall of the intestine (including Peyer's patches), the skin and the blood showed no labelled cells. This was also true of all autoradiograms of the organs taken from the control animals that had been injected with dead cells and H^3-thymidine. The number of grains per field of vision over these sections was about the same as the number outside the sections, i.e. the so-called background grains. The number of background grains was about the same in all autoradiograms, about 1–2 grains per 1000 μ^2.

Discussion

Several assumptions may be made concerning the nature of the labelled lymphocytes in the spleen, liver, mesenteric lymph node, lung and bone marrow. They may be: (1) the transfused lymphocytes, (2) lymphocytes generated by mitosis of the transfused lymphocytes, (3) lymphocytes that have incorporated isotope injected together with the transfused cells, (4) lymphocytes that have incorporated isotope released from the transfused cells and (5) lymphocytes produced by a transformation of labelled reticular cells or phagocytes.

The last-mentioned alternative, which most probably applies to only a very small proportion of the lymphocytes observed, is discussed in Chapter VII. As to alternatives (3) and (4), these are very unlikely in the light of the following facts. When dead cells as well as the same quantity of H^3-thymidine as was used for labelling were injected, no labelled cells were found in any of the organs examined. The quantity of H^3-thymidine injected with the cells is thus too small to give rise to any labelled cells in these organs. The same applies to the isotope released from the dead cells and very probably also to that released from the living ones. It can be assumed that most of the isotope in the cells was incorporated in the DNA (*Schooley, Bryant & Kelly* 1959, *Pelc & La*

Cour 1959 and *Crathorn & Shooter* 1960), and except in the case of mitosis a turnover of the DNA of the living cells apparently does not need to be taken into account (*Hughes, Bond, Brecher, Cronkite, Painter, Quastler & Sherman* 1958 and *MacDonald & Mallory* 1959). Additional support for the assumption that the transfused lymphocytes or lymphocytes generated by mitosis of these have been registered is that the degree of labelling of the cells in the sections of the spleen is much the same for all the animals, while the number of labelled cells per 100 fields of vision varies considerably (see Table 2). Supposing lymphocytes incorporating isotope injected together with the transfused cells or isotope released from these cells had been registered, one would expect the degree of labelling of the lymphocytes to be in better agreement with the number of labelled cells per 100 fields of vision in the spleen. No certain difference in the degree of labelling could be observed between the lymphocytes in this and the remaining organs cut into sections, an indication that this argument holds true everywhere.

In the smears of bone marrow, spleen and lymph node the majority of labelled lymphocytes had about as many grains as those in the thymus cell suspensions, an indication that they are indeed the transfused cells. Some cells contained a lesser quantity of isotope, possibly because they were formed by mitosis of the transfused cells. Another explanation could be that some of the isotope at the time of transfusion was subsequently released. From the above-mentioned indications it may be concluded that the overwhelming majority of the lymphocytes registered really are those transfused and possibly some formed by mitosis of those transfused.

Of the organs examined the spleen contained the greatest number of labelled lymphocytes per 100 fields of vision. A considerably lower frequency was shown by the autoradiograms of the liver, but on account of the size of this organ it is probable that a relatively large part of the transfused cells accumulates here. It is impossible to determine the order in which spleen and liver should be placed as regards their capacity to take up the transfused lymphocytes. Only a very small part of each organ, especially of the liver, has been examined, and that part may not reflect the frequency of labelled cells in the entire organ. However, considerably fewer lymphocytes were recovered in the mesenteric lymph

node, lung and bone marrow, and it is very probable that a much smaller proportion of the transfused lymphocytes find their way to these organs.

It is also very difficult to determine how many of the transfused, labelled lymphocytes had reached the organs examined, partly since we know too little about the efficiency of the autoradiographic film. Very rough estimates suggest that on the average 20% were present. To what extent the remaining cells were located in other organs or had been destroyed is altogether a matter of conjecture.

The question as to how far the results obtained really reflect the distribution of the thymus lymphocytes under physiological conditions will be discussed in Chapter VII. In the same Chapter the present results are compared with results of earlier transfusion experiments using other methods and the question is raised to what extent the number of grains over the cells represents a measure of their isotope content.

Summary

The fate of transfused thymus lymphocytes labelled *in vitro* with H^3-thymidine was studied in guinea-pigs. Eight hours after the autologous transfusion the animals were killed, and spleen, liver, mesenteric lymph node, lung, intestine, skin, blood and bone marrow were preserved for autoradiographic examination. It was shown that the spleen and the liver contained most labelled lymphocytes, while relatively few could be observed in the mesenteric lymph node, lung and bone marrow. In the spleen the lymphocytes were detected mainly in the red pulp, and in the liver most of them were found in the lobules. In the lymph node they were diffusely spread over both medulla and cortex with the exception of the follicles, in which only stray ones were found. In the lung the lymphocytes were traced to the alveolar walls. No labelled lymphocytes were recovered in the wall of the intestine, the skin or the blood. No support was obtained for the theory that lymphocytes can turn into other types of cells.

That the overwhelming majority of the labelled lymphocytes observed almost certainly were the transfused lymphocytes or lymphocytes formed by mitosis of these cells was shown on the one hand by transfusions of dead cells and on the other by comparisons of the degree of labelling of the lymphocytes.

CHAPTER III

The Fate in Guinea-Pigs of Transfused Thymus Lymphocytes Labelled *in vitro* with S^{35}-Methionine

In the investigation reported in the previous Chapter thymus lymphocytes labelled *in vitro* with H^3-thymidine were transfused. Only about 10% of the lymphocytes were labelled, presumably mainly those synthesizing DNA. The question is to what extent all the other thymus lymphocytes are distributed after transfusion in the same way as those labelled. There are distinct morphological differences between various thymus lymphocytes but to what degree there also are differences concerning the function and therefore probably the distribution of the cells after leaving the blood is unknown.

Fichtelius (1958 *a* and *b*) has investigated possible differences between the distribution of transfused thymus lymphocytes and lymph node lymphocytes. This question was also studied by means of apposition autoradiography (*Diderholm & Fichtelius* 1959 *b*). These investigations have revealed certain differences between these types of cells.

The aim of the present work is to study to what extent different types of thymus lymphocytes are differently distributed after transfusion. Thymus lymphocytes are labelled *in vitro* with S^{35}-methionine, transfused and registered in the way described in Chapter II. The S^{35}-autoradiograms are compared with the H^3-autoradiograms obtained in the previous experiment. As will be shown below, a wider selection of lymphocytes is labelled by S^{35}-methionine than by H^3-thymidine.

Material and methods

20 male guinea-pigs, about 4 weeks old and weighing 156–237 g, were used. They were of the same strain as the animals employed in the previous investigation and were given the same diet.

The excision of the thymus and preparation of the cell suspension were carried out the way described in Chapter II. When the volume of the suspension had been adjusted to 0.9 cc, S^{35}-DL-methionine[1] that had been dissolved in physiological saline was added. At the beginning of the experiment the methionine had a specific activity of 31.3 mC per mmole and 0.05 cc contained 12 μC. This volume was added to all the suspensions. The whole experiment took 9 days, and since no attention was paid to the fact that the specific activity of the methionine decreased somewhat during that time, the quantity of the isotope added to some of the suspensions was actually a little less than 12 μC. Instead the films of the organs from the animals given these suspensions were exposed for a longer period.

The suspensions were kept in a water-bath at 37°C for 60 minutes. In 8 suspensions to be injected into the control animals the cells were killed by heating as described in Chapter II. An account of the number and types of lymphocytes labelled is given below.

In this investigation, too, *Schrek's* (1943) test was used to determine the percentage of dead cells. The results were similar to those obtained when the H^3-labelled cells were tested.

The autologous transfusions were carried out in the way described in Chapter II. Altogether 20 transfusions were given. Each of 8 control animals was injected with suspension heated after the labelling and with 0.05 cc of the solution containing S^{35}-methionine. The number of cells injected into the experimental animals was on the average ca 12.9×10^7 (ca $5.6 \times 10^7 - 21.4 \times 10^7$) and into the control animals ca 8.5×10^7 (ca $4.7 \times 10^7 - 12.7 \times 10^7$). The transfusion gave no after-effects in any of the animals.

The animals were killed 8 hours after the transfusion in the same way and at the same time of day as in the investigation described in Chapter II. The same organs were dissected out and fixed according to the same method. Smears were made of the thymus cell suspension, blood and bone marrow and of cell suspensions from pieces of the lymph node and spleen. These were treated as described in Chapter II. The subsequent treatment of the organs and the autoradiographic technique were the same as before, an exception being the thickness of the sections, which in the present case was 8 μ.

The exposure time was 48–68 days. The films were developed in Kodak D 19 b for 6 minutes at 18–20°C. Rinsing in water and fixation were carried out as previously described. As a rule about 20 sections of each organ were autoradiographed. In the case of the spleen at least 35 sections were examined, however. From every animal about 7 smears each of the blood, bone marrow and of the prepared suspensions were autoradiographed. Both smears and sections were stained the same way as in the previous investigation.

The autoradiograms were examined as described before. For a cell to be considered labelled at least 10 grains were required over it, and

[1] Supplied by the Radiochemical Centre, Amersham, England.

TABLE 3. *Percentage of S^{35}-labelled lymphocytes in the spleen observed in the red pulp, mean grain count over the labelled lymphocytes and mean number of these cells per 100 fields of vision in the spleen where 12 determinations were made.*

Determination no.	Percentage of labelled lymphocytes in red pulp	Mean grain count over labelled lymphocytes	Mean number of labelled lymphocytes per 100 fields of vision
1	95.8	19.5	191.6
2	93.6	20.0	199.2
3	93.9	20.6	200.4
4	91.5	20.4	177.3
5	92.7	20.5	193.4
6	93.7	19.9	192.3
7	90.9	20.1	182.8
8	91.5	20.2	189.0
9	94.3	19.9	201.6
10	90.0	20.3	189.8
11	91.5	20.7	201.2
12	91.3	20.5	170.4
Mean and standard deviation	92.6±1.7	20.2±0.4	190.7±9.8
Coefficient of variation	±1.8	±1.7	±5.2

this applied to smears as well as sections. In the spleen, which was more closely examined than the other organs, the position of 1000 labelled lymphocytes was determined. The number of grains over every 3rd cell of these 1000 was counted and the mean number of labelled lymphocytes per 100 fields of vision was determined. As in the previous investigation, one field of vision corresponded to an area of about 7.8×10^3 μ^2 of the section. In this investigation, too, 12 such determinations were made from one and the same spleen in order to get an idea of the random error. The results of these 12 determinations are listed in Table 3.

RESULTS

The autoradiograms of the thymus cell suspensions showed that about 40–55% of the cells were labelled. Almost all of these were classified as lymphocytes, the rest being mainly reticular cells and granulocytes. Lymphocytes of all sizes were labelled, and no great

TABLE 4. *Percentage of S^{35}-labelled lymphocytes in the spleen observed in the red pulp, mean grain count over the labelled lymphocytes and mean number of these cells per 100 fields of vision in the spleen.*

Animal no.	Percentage of labelled lymphocytes in red pulp	Mean grain count over labelled lymphocytes	Mean number of labelled lymphocytes per 100 fields of vision
1	92.6	20.2	190.7
2	96.2	18.2	118.8
3	91.7	19.3	84.8
4	95.4	18.2	152.9
5	92.3	19.3	47.8
6	95.1	18.7	86.4
7	91.0	18.9	75.5
8	92.5	22.1	23.8
9	88.8	21.1	21.1
10	94.7	18.1	91.8
11	92.9	20.6	47.5
12	90.2	18.5	44.2
Mean and standard error	92.8±0.6	19.4±0.4	82.1±14.9
Coefficient of variation	±2.4	±6.6	±62.9

differences in labelling were observed between large, medium and small lymphocytes. Occasionally, however, the percentage of labelled cells was somewhat higher in the classes of large and medium cells than in the class of small cells, and the number of grains over the latter was usually somewhat smaller than over the remaining cells. As in the previous investigation about 95–98% of the cells in the suspensions were lymphocytes, and about 60–85% of these were small. The three size classes are defined in Chapter II. Plate 5 shows labelled lymphocytes from a thymus cell suspension.

The autoradiograms of the spleens showed a rather high frequency of labelled lymphocytes in almost all animals. A mean number of 92.8% of the cells were situated within the red pulp, and their frequency per field of vision was greater there than in the white pulp. Occasionally the lymphocytes were concentrated to regions near the white pulp in the same way as the cells labelled

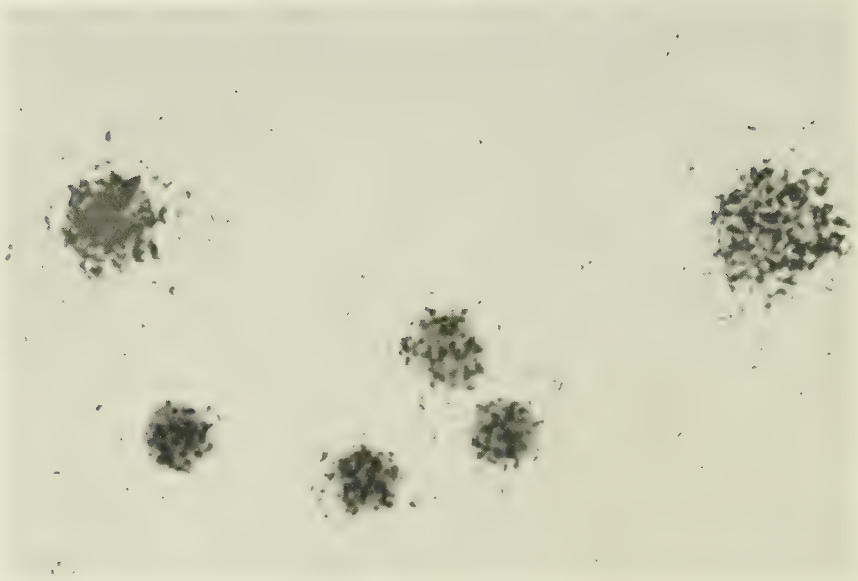

Plate 5. Autoradiogram of a smear from a thymus cell suspension labelled *in vitro* with S^{35}-methionine. All six cells are lymphocytes and all are labelled. Some background grains are seen.
(Giemsa, × 1400.)

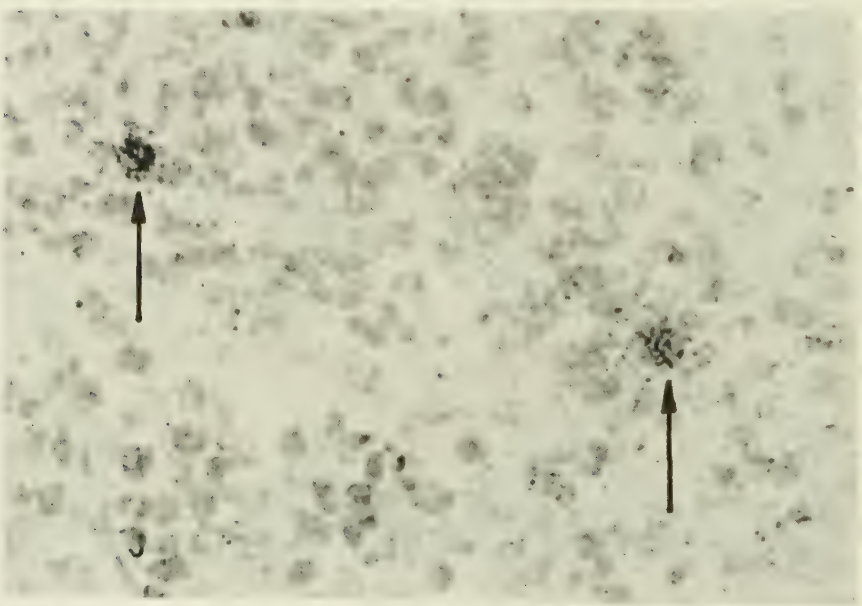

Plate 6. Autoradiogram of a section from a spleen showing two S^{35}-labelled lymphocytes in the red pulp. Eight hours after the autologous transfusion.
(Haematoxylin, × 850.)

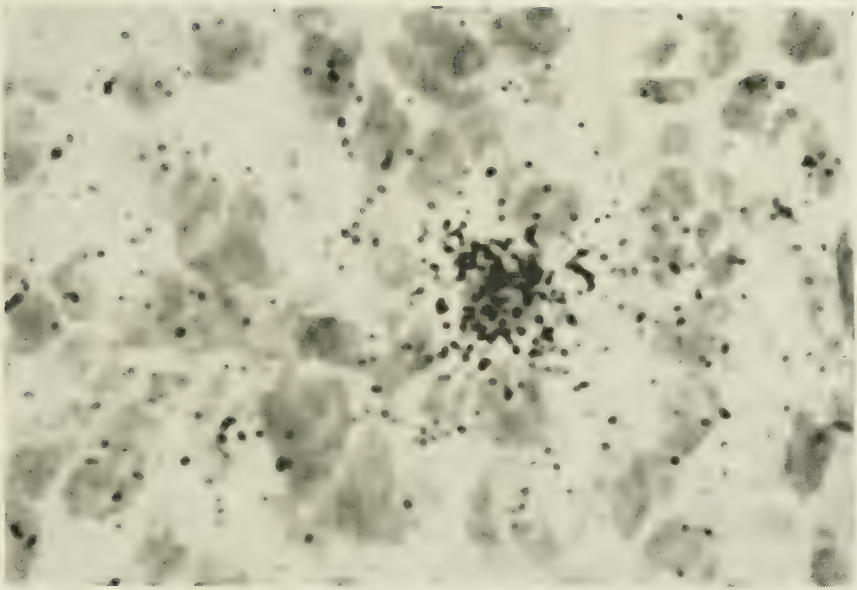

Plate 7. Highly magnified S^{35}-labelled lymphocyte in the red pulp of the same spleen as in Plate 6. The resolving power of the autoradiogram is not so high as with cells labelled with H^3. In contrast to the essential localization of grains directly over H^3-labelled cells grains from S^{35}-labelled cells are more diffusely distributed, and, as seen in the Plate, some may be as much as a cell diameter away from the cell.
(Haematoxylin, × 1800.)

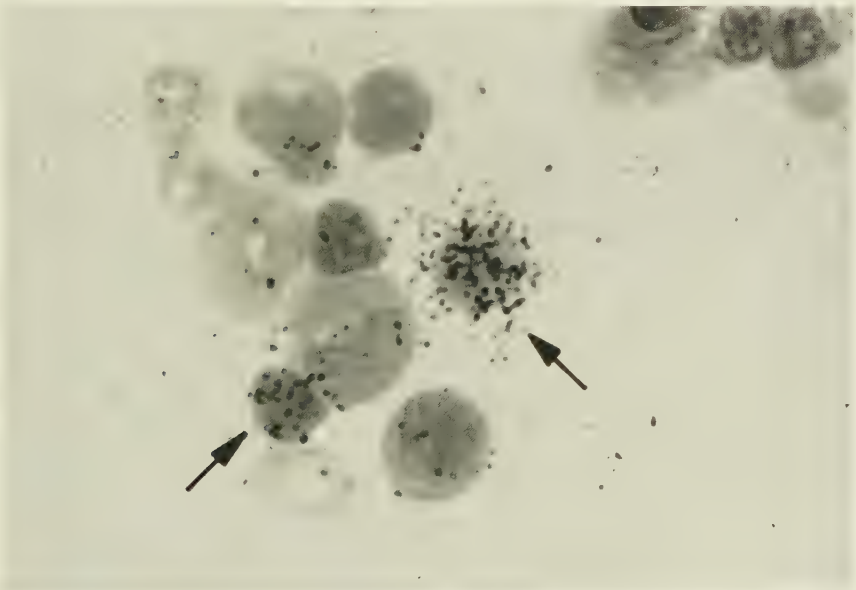

Plate 8. Autoradiogram of a bone marrow smear showing two S^{35}-labelled lymphocytes. Eight hours after the autologous transfusion. Some background grains are seen.
(Giemsa, × 1400.)

with H^3. In this investigation, too, no difference was observed between the frequencies of labelled cells per field of vision in the perifollicular zones, the germinal centres and the other parts of the white pulp. Table 4 shows the percentages for all the animals. Existing variations are rather small. The Table also shows the mean numbers of grains counted over the labelled lymphocytes and the mean numbers of labelled lymphocytes per 100 fields of vision. For the individual animals the mean grain count over the labelled lymphocytes varied between 18.1 and 22.1, the mean for the whole material being 19.4. The mean number of labelled lymphocytes per 100 fields of vision was for the individuals 21.1–190.7, for the whole material 82.1.

Most of the labelled cells in the spleen were classified as lymphocytes. The remainder comprised reticular cells and cells that could not be identified. No labelled plasma cells were observed, however. As in the previous investigation, no difference in distribution was found between labelled lymphocytes and other labelled cells. Plates 6 och 7 show labelled lymphocytes in the spleen.

In all the animals labelled lymphocytes were also found in the liver. The frequency per 100 fields of vision of these cells was considerably lower than in the spleen of the animal concerned, however, only about 1/10–1/20. Most of the lymphocytes were diffusely spread within the lobules, but occasionally one was found in the interlobular connective tissue. Besides the lymphocytes there were some other labelled cells. These could not be identified, but their distribution agreed with that of the labelled lymphocytes.

Labelled lymphocytes were also found in the mesenteric lymph node and lung of all the animals. However, the frequency of these per 100 fields of vision was considerably lower than in the spleen and liver of the same animal. In the mesenteric lymph node they were diffusely spread over both medulla and cortex with the exception of the follicles, in which only stray ones were found. In the lung the labelled lymphocytes were observed in the alveolar walls. In both these organs, too, there were some labelled cells that could not be classified. The distribution of these within the organ appeared to agree with the distribution of the labelled lymphocytes. In all the organs cut into sections the labelled cells practically always occurred singly, exceptionally in twos or threes.

In all the animals the bone marrow contained rather few labelled lymphocytes. Occasionally labelled reticular cells were found as

well as some labelled cells that could not be identified. Plate 8 shows an autoradiogram of a bone marrow smear.

Concerning the degree of labelling of the lymphocytes no certain difference between the various organs appeared from the autoradiograms of the sections. When the autoradiograms of the smears were compared, it was shown that the lymphocytes in the thymus cell suspensions were usually more strongly labelled than those in the other smears. As a rule almost twice as many grains were found over the lymphocytes in the thymus cell suspension as over those in the other smears.

No labelled cells were revealed by the autoradiograms of the wall of the intestine (including Peyer's patches), the skin and the blood. This was also the case for the organs of the animals that had received dead cells and S^{35}-methionine. The number of grains over these sections was about the same as or somewhat higher than the number of background grains. The latter was higher than in the previous investigation, namely about 10–15 grains per 1000 μ^2. The autoradiograms were found to vary slightly in background intensity.

Discussion

The first question to be answered here is how far the labelled lymphocytes detected in the spleen, liver, mesenteric lymph node, lung and bone marrow may be regarded as those originally transfused. The alternatives discussed in Chapter II regarding the nature of the lymphocytes labelled with H^3 apply to the S^{35}-labelled ones as well. In the organs of the control animals no labelled cells were discovered, showing that neither the quantity of S^{35}-methionine injected together with the cells nor the isotope that was probably released from the dead cells could give rise to labelled cells in the organs examined. Presumably the S^{35}-methionine was largely incorporated in proteins and part of the isotope of the living cells may have been released in the course of the experiment. It is unlikely, however, that this radioactivity gave rise to labelled cells, since none were found in the controls. The mean grain count of the lymphocytes in the sections of the spleen was about the same for all the animals, while the mean number of labelled cells per 100 fields of vision varied considerably, another indication favouring the assumption that the labelled lymphocytes actually are the trans-

fused cells or such formed by mitosis of these cells (see Table 4). Here, as in the previous investigation, no certain difference was observed between the degrees of labelling of the lymphocytes in the spleen and in other organs cut into sections.

Comparisons between the degrees of labelling of the individual labelled lymphocytes in the smears of thymus, spleen, lymph node and bone marrow usually showed the radioactivity of the lymphocytes in the thymus to be almost twice as high as that of the others, another indication that the lymphocytes registered are the transfused cells or such formed by mitosis of these cells. If the isotope of the transfused cells had been released and incorporated into other lymphocytes, there should have been greater differences in the isotope content of the cells. The probable reasons for the difference observed are that some cells had divided and that part of the radioactivity had been released from the cells after the transfusion. Summing up the evidence it seems extremely likely that almost all the labelled lymphocytes in the spleen, liver, mesenteric lymph node, lung and bone marrow are the transfused cells or such formed by mitosis of these.

Of all the organs examined the spleen contained the greatest number of labelled lymphocytes per 100 fields of vision. The autoradiograms of the liver showed considerably fewer cells. As was the case with the transfused lymphocytes labelled with H^3 it is impossible to establish the order in which spleen and liver should be placed with regard to the total content of transfused lymphocytes. Concerning the mesenteric lymph node, lung and bone marrow, however, it appears fairly certain that only a few transfused cells find their way there.

A comparison between the distribution of the lymphocytes labelled with S^{35} and those labelled with H^3 showed no certain differences. The autoradiograms of the smears of the thymus cell suspensions revealed that S^{35} labelled no less than about five times as many cells as H^3. However, in the sections of the spleens the number of lymphocytes labelled with S^{35} per 100 fields of vision was on the average only about twice as great as the number of H^3-labelled lymphocytes. The question arises whether the number of S^{35}-labelled cells in the spleen ought not to be greater if lymphocytes labelled with H^3-thymidine and S^{35}-methionine are distributed in the same way after transfusion. Even if the exact number of labelled cells injected, the size of the spleens and the thickness of the sections

are taken into account it is impossible to make an accurate comparison, partly since we do not know if the ratios between the efficiencies of the autoradiographic films of the smears and sections have been the same for H^3 and S^{35}. In the investigations reported here this has most probably not been the case. It is moreover likely that the lymphocytes give off more S^{35} than H^3 in the course of the experiment, during fixation and the subsequent treatment of the organs. For these reasons it is only possible to compare the relative number of labelled lymphocytes in the organs and the distribution of the cells within these.

Both H^3- och S^{35}-labelled lymphocytes could be traced principally to the spleen and liver and to a lesser extent to the mesenteric lymph node, lung and bone marrow. In the spleen an average of over 90 % of the cells had accumulated in the red pulp. The mean percentage for the cells labelled with H^3 was 91.0 ± 0.5 and for those labelled with S^{35} 92.8 ± 0.6. This difference was not considered statistically significant ($P > 0.02$). In the remaining organs, too, no certain differences were observed between the distribution of the H^3- and S^{35}-labelled lymphocytes. It must therefore be concluded that in both investigations the transfused labelled lymphocytes have behaved in more or less the same way.

It may now be asked whether the type of lymphocytes registered in the two investigations was on the whole the same. In the autoradiograms of the smears of the thymus cell suspensions many more S^{35}- than H^3-labelled lymphocytes were seen. Further, most of the cells labelled with S^{35} were small, while the majority of the H^3-labelled ones were medium-sized. This strongly indicates that the cell-populations were not identical in both cases. Presumably it would be possible to label some of the cells incorporating S^{35}-methionine with H^3-thymidine as well, provided the quantity of H^3-thymidine was the same as in the investigation in Chapter II. Most probably this does not apply to more than rather few of the cells, however.

Here, as in Chapter II, the question arises how great a part of the transfused, labelled lymphocytes was present in the organs examined. According to a very rough estimate there were on the average 12 %. In this case the proportion of labelled lymphocytes recovered thus appears to be smaller than in the previous investigation. The most obvious explanation for this is that in course of the experiment and during the treatment of the organs before

the film was applied S^{35} was probably released from the cells more easily than H^3.

The problem whether transfusion experiments can shed light on the migration of the thymus lymphocytes to other organs under physiological conditions is discussed in Chapter VII, where the present results are also compared to those obtained in earlier transfusion experiments. Moreover the question is raised to what extent the number of grains over the cells represents a measure of their isotope content.

Summary

Thymus lymphocytes were labelled *in vitro* with S^{35}-methionine and transfused to guinea-pigs. Eight hours after the autologous transfusion the animals were killed and the content and distribution of labelled lymphocytes in the spleen, liver, mesenteric lymph node, lung, wall of the intestine, skin, blood and bone marrow were studied by means of autoradiography. The autoradiograms showed that the spleen and liver contained most labelled lymphocytes, while relatively few were discovered in the mesenteric lymph node, lung and bone marrow. Most of the lymphocytes in the spleen were observed in the red pulp, while in the liver the majority were found in the lobules. In the mesenteric lymph node they were diffusely spread over both medulla and cortex with the exception of the follicles, in which only stray ones were found. In the lung the lymphocytes were observed in the alveolar walls. No labelled cells were detected in the wall of the intestine, the blood and the skin. No support was obtained for the theory that lymphocytes can be transformed into other types of cells.

By means of control experiments with dead cells on the one hand and comparisons between the degrees of labelling of the lymphocytes on the other, it was shown that in all probability nearly all the registered lymphocytes were the transfused cells or such formed by mitosis of these cells.

A comparison with the results of the transfusion experiment with lymphocytes labelled with H^3-thymidine shows that both types of lymphocytes are distributed to the same organs, where they accumulate in more or less the same regions.

CHAPTER IV

The Fate in Immunized Guinea-Pigs of Transfused Thymus Lymphocytes Labelled *in vitro* with H^3-Thymidine

In the investigations described in Chapters II and III as well as in other works it has been shown that transfused lymphocytes accumulate to a large extent in the spleen (references in Chapter VII). What subsequently happens to them there is not clear, but one of many possibilities is that they turn into plasma cells. According to the hypothesis by *Fichtelius* (1953, 1957 *c* and 1960), this is supposed to be the case for thymus lymphocytes, but in the transfusion experiments described in Chapters II and III no labelled plasma cells indicating such a transformation have been observed.

The supposed ability of lymphocytes to turn into plasma cells has been the subject of many investigations. *Downey* (1911), *Bloom* (1928), *Jordan* (1954) and others have observed transitional forms between lymphocytes and plasma cells, which they consider to be evidence for such a transformation. *Neil & Dixon* (1959) injected lymph node cell suspensions from immunized rabbits subcutaneously and intramuscularly into other rabbits. The sites of injection were examined and many plasma cells were observed. The authors suggest that some of these were derived from the injected lymphocytes. The ability of thoracic duct lymphocytes to turn into other types of cells was studied by *Schooley & Berman* (1960). The cells were labelled with H^3-thymidine and cultivated together with unlabelled bone marrow cells in diffusion chambers implanted into the peritoneal cavity. Labelled plasma cells were observed, but the authors did not think that these had necessarily developed from the lymphocytes.

The present investigation consists of transfusion experiments to study the ability of thymus lymphocytes to turn into plasma cells. Labelled thymus lymphocytes are transfused to guinea-pigs previously immunized with *S. typhi* to stimulate plasma cell formation. The spleen and mesenteric lymph node are examined at different

intervals after the transfusion by means of autoradiography. In the first place the autoradiograms are studied for the presence of labelled plasma cells. The results are further compared to those of the investigations described in Chapters II and III in order to find out whether the distribution of the transfused cells differs in any way.

Material and methods

Autologous transfusions of thymus lymphocytes were made under secondary response to *S. typhi*. Male guinea-pigs of the strain used in the previous investigations were employed and they were given the same diet. When the animals were about 3 weeks old and weighed 158–210 g, primary immunization was carried out as follows: equal volumes of 2% agar at 50°C and physiological saline containing about 10^9 formalin-killed *S. typhi* (strain I.S. 57) per cc were mixed in a syringe and injected subcutaneously. The animals received 0.2 cc of the mixture per 100 g body weight. After 19 days a booster dose was given. 18 cc physiological saline containing about 10^9 formalin-killed *S. typhi* per cc were centrifuged for 30 minutes at 3000 r.p.m. and 12 cc of the supernatant fluid pipetted off. The rest was stirred up and 0.2 cc per 100 g body weight was injected into the deep jugular vein.

The preparation of the thymus cell suspension and the labelling of the cells were carried out in the way described in Chapter II except that 0.8 μC H^3-thymidine[1] was added to each suspension. The specific activity of the thymidine was 360 mC per mmole and it had been dissolved in physiological saline so that 0.1 cc contained 0.8 μC. The cells were labelled in the type of glass beaker used in the previous investigations, and after thymidine had been added the volume of the cell suspension was 1 cc. As before the labelling time was 60 minutes. In 8 suspensions to be injected into control animals the cells were killed by heating as described in Chapter II.

18 and 42 hours after the administration of the booster dose the cells were transfused in the same way as in previous investigations. 0.6 cc of the suspension was injected. The number of cells injected into the experimental animals was on the average ca 11.0×10^7 (ca $2.6 \times 10^7 - 29.0 \times 10^7$) and into the control animals ca 13.3×10^7 (ca $8.5 \times 10^7 - 20.3 \times 10^7$). The transfusion caused no after-effects in any of the animals.

The animals were anaesthetized $3\,^1/_2$ and $5\,^1/_2$ days later by inhaling ether for a short period. The wound was cut open and about 3 cc blood taken from the deep jugular veins. Immediately afterwards they were killed by a blow on the neck. This was done at the same time of day as in the previous investigations.

[1] Supplied by Schwartz Laboratories Inc., New York.

Altogether 20 transfusions were made. 5 animals got their transfusion 18 hours after the booster dose and were killed $3^1/_3$ days later. 2 of these animals received 0.8 μC H^3-thymidine dissolved in 0.1 cc physiological saline apart from cell suspension heated after the labelling. Another group of the same size was treated the same way but killed $5^1/_3$ days after the transfusion. The remaining 10 animals got the booster dose 42 hours before the transfusion. 5 of these were killed $3^1/_3$ days, the others $5^1/_3$ days after the transfusion. 2 animals in each group received 0.8 μC H^3-thymidine and suspension heated after labelling.

The spleen and the mesenteric lymph node situated at the main stems of the mesenteric vessels were dissected out and prepared for autoradiographic examination. After being fixed in a mixture of alcohol, formalin and acetic acid according to *Serra* (1946) they were embedded in paraffin and sections 6 μ thick were cut. Smears were made of the thymus cell suspensions and these were treated as described in Chapter II.

The autoradiographic technique was that employed in the previous investigations and the same type of film was used. The exposure times were 30 days for the smears and 69–72 days for the sections. The films were developed in Kodak D 19 b for 6 minutes at 18–20°C. Fixing and rinsing in water were carried out the way described in Chapter II. The smears were stained with Giemsa and the sections with methyl green–pyronin according to Unna–Pappenheim. 3 smears of each suspension and at least 80 sections from each organ were examined. A minimum of 3 grains was required over a cell in a smear for it to be considered labelled; in a section 5 grains were required. As in the previous investigations, one field of vision corresponded to an area of about 7.8×10^3 μ^2 of the section.

The reliability of the technique of immunization was tested in 8 animals, the antibody content in serum being determined by agglutination of H antigen. (This was done by Ass. Prof. G. Laurell, Institute of Bacteriology, Uppsala.) The serum was diluted serially and an equal amount of the antigen was added. The tubes were then incubated in a water-bath at 37°C and readings were made the following day. The last tube in the series showing agglutination visible to the naked eye was registered as the agglutination limit. 2 animals had the titre of 1/20480, 1 had 1/10240, 3 had 1/2560 and 2 had 1/1280.

Results

The thymus cell suspensions consisted of about 95–98% lymphocytes and contained the same frequency of labelled cells as the suspensions in the investigation described in Chapter II, i.e. about 7–12%. Almost all of these were lymphocytes, but a few reticular cells and some unidentifiable cells were also observed. Most of the

labelled lymphocytes were medium-sized, the rest being either large or small. The three size classes are defined in Chapter II.

The autoradiograms of the spleens of all the animals showed labelled cells, but the number of these per 100 fields of vision was much lower than that of the spleens described in Chapter II. Almost all the labelled cells were classified as lymphocytes, the rest being reticular cells and a few cells that could not be identified. All spleens contained a large number of plasma cells, none of which were labelled, however. About 50–80% of the labelled lymphocytes were observed in the red pulp, where, as in the white pulp, it seemed they were evenly distributed. Thus the accumulations in regions near the white pulp noted in earlier investigations could not be observed in the present case.

The mesenteric lymph node of a few animals only contained labelled cells, practically all of which were classified as lymphocytes. The remainder consisted of cells that could not be identified. As in the case of the spleen many plasma cells were observed, none of which were labelled, however. In the lymph node the labelled lymphocytes were diffusely spread over both medulla and cortex with the exception of the follicles, in which only stray ones were occasionally found. In the lymph node as well as the spleen the labelled cells always occurred singly.

The mean number of grains over each labelled cell was about 10. In this respect as well as regarding the frequency and distribution of the labelled lymphocytes in the organs examined it seemed to make no difference at what time the animals had received their booster dose and when they had been killed.

The autoradiograms of organs from animals that had received dead cells and H^3-thymidine showed no labelled cells and the number of grains over the sections was of the same order as the background intensity. In the sections the latter was higher than in the investigation described in Chapter II, i.e. about 8–10 grains per 1000 μ^2. In the smears the number of background grains was about 2–3 per 1000 μ^2.

Discussion

In Chapter II we discussed whether the H^3-labelled lymphocytes in the organs examined were identical with the transfused cells. The following points were considered to indicate strongly that al-

most all the lymphocytes registered were the transfused lymphocytes or lymphocytes formed by mitosis of these: (1) No labelled cells were found when dead cells plus a quantity of H^3-thymidine equal to that used in labelling hade been injected. (2) The variations in the degree of labelling of the cells were rather slight, while the number of labelled cells per 100 fields of vision in the organs varied considerably. (3) The labelled cells in the smears of thymus, spleen, lymph node and bone marrow were labelled to more or less the same degree. In the present investigation the last two conditions have not been examined. However, the fact that the autoradiograms of the organs of the control animals showed no labelled cells at all strongly indicates that the bulk of the labelled cells actually are those transfused or are cells formed by mitosis of these.

No labelled plasma cells could be observed either in the spleen or in the mesenteric lymph node. This does not prove, however, that thymus lymphocytes are unable to differentiate into plasma cells. If the antigen had been another or if immunization had been carried out at a different time, the results might have been otherwise. It is also possible that, while H^3-labelled lymphocytes are unable to turn into plasma cells, other types of thymus lymphocytes can.

Since the lymphocytes recovered were so few, especially in the mesenteric lymph node, it is impossible to determine whether their distribution among the organs differed in any way from that in the investigations described in Chapters II and III. Their distribution within the spleen did, however, differ in the present case. About 50–80 % of the labelled lymphocytes were found in the red pulp, whereas about 90 % were traced to this pulp in the previous investigations. Since in the spleen, too, the number of lymphocytes recovered was small, about 300–900 in each specimen, it is impossible to establish the existence of a real difference concerning the distribution of lymphocytes within this organ. Most probably the difference actually is real, but whether this is due to the immunization it is impossible to determine.

The cells observed were rather faintly labelled in comparison with the transfused H^3-labelled cells in Chapter II. The most probable explanation for this is that many cells had divided, probably several times. Other possibilities such as that the more strongly labelled cells were killed by the radioactive isotope or that they

were distributed to other organs cannot be excluded, however. The last two factors can also explain why rather few labelled cells were found in the organs examined. What is more probable in this respect, however, is that many cell divisions have taken place, causing the isotope content to decrease to such an extent that it can no longer be registered by the film.

Summary

Autologous transfusions of thymus lympocytes labelled *in vitro* with H^3-thymidine were carried out on guinea-pigs that had been immunized with *S. typhi*. The tranfusions took place under secondary response 18 and 42 hours after the administration of the booster dose. The animals were killed $3\,^1/_3$ and $5\,^1/_3$ days after the transfusion, and spleen and mesenteric lymph node were examined by means of autoradiography.

Autoradiograms of the spleen and lymph node showed labelled lymphocytes. No labelled plasma cells were observed, however, and thus no support was obtained for the hypothesis that thymus lymphocytes are able to turn into plasma cells. In the spleen about 65% of the labelled lymphocytes were localized in the red pulp, i.e. a smaller proportion than in the investigations with non-immunized guinea-pigs (Chapters II and III). In the mesenteric lymph node the lymphocytes were diffusely spread over both medulla and cortex with the exception of the follicles, in which only stray cells were occasionally found.

By means of control experiments with cells killed after labelling it was shown that the bulk of the labelled cells observed were very probably the transfused cells or cells formed by mitosis of these.

CHAPTER V

The Influence of Subtotal Thymectomy on the Spleen and Lymph Nodes in Immunized and Non-Immunized Guinea-Pigs (Weights, Volumes of Red and White Splenic Pulps and Plasma Cell Content of the Spleen)

The transfusion experiments in Chapters II, III and IV and other transfusion experiments with thymus lymphocytes have shown that some of the cells accumulate in the spleen and lymph nodes (references in Chapter VII). The question to what extent the migration of thymus lymphocytes to these organs is greater in immunized than in non-immunized animals has been studied in some of these investigations, but no definite answer has been found. The investigations all have certain disadvantages when it comes to studying what organs thymus lymphocytes migrate to under physiological conditions. The quantity of cells injected over a short period is rather large, and it may be that under physiological conditions some would actually never leave the thymus. Another drawback is that the cells may be damaged when the cell suspension is prepared, during transfusion and by the radioactive isotope or fluorescent dye.

If the lymphocytes in the thymus are released to the blood and then migrate to the spleen and lymph nodes, it probably follows that these organs are affected by thymectomy. Unless a compensatory mechanism is developed, the weights of these organs ought to be lower in the ectomized animals than in the controls, and according to the results of the investigations described in Chapters II and III the red pulp of the spleen, should in the non-immunized animals decrease relatively more than the white pulp. If the migration of thymus lymphocytes to the spleen and lymph nodes is greater in immunized than non-immunized animals, it is possible that thymectomy after immunization affects these organs to a greater extent. According to *Fichtelius'* (1953, 1957 c and 1960) hypothesis concerning the part played by the thymus in the formation of antibodies, thymectomy ought to cause a decrease in the plasma cell content of the spleen.

Investigations in which the spleen and lymph nodes were examined after thymectomy have been reported in a number of papers. A survey of the earlier works on this subject is given by *Gyllensten* (1953). In many cases the procedure included weighing the organs and determining the volumes of the red and white splenic pulps. It is, however, difficult to draw any definite conclusions from the results obtained, since the material was usually too small. Moreover the animals used were more often than not of species in which the thymus is situated intrathoracically. On such animals thymectomy has a considerable stress effect, and in most investigations the controls were not sham-operated. A study of the influence of subtotal thymectomy on the weights of lymph nodes and spleen was made by *Gyllensten* (1953), who used guinea-pigs. On account of the shallow position of the main part of the thymus tissue on the throat subtotal thymectomy could be carried out on these animals without causing them stress of any significance. Fourteen days later *Gyllensten* found that the weights of the lymph nodes were higher in thymus-resected animals than in sham-operated controls. This was not the case for the spleen, where no certain differences were obtained. In an investigation by *Schooley & Kelly* (1958) the lymph nodes of thymectomized rats weighed less than the same organs from sham-operated controls. *Bierring* (1960), on the other hand, found no differences between thymectomized and sham-operated rats concerning the weights of spleen and lymph nodes. *Hammar* (1938) immunized rabbits with *S. paratyphi* B, thymectomized them and examined the spleen and lymph nodes. He found that the organs weighed less in the ectomized animals than in the controls, which had not been sham-operated, however. One explanation for his and *Schooley and Kelly's* results may be that thymectomy stops a possible flow of lymphocytes from the thymus to the spleen and lymph nodes. The results obtained by the other authors may be explained by a compensatory mechanism.

In order to examine further the effect of thymectomy on the spleen and lymph nodes and thus to attack the problem of the migration and transformation of thymus lymphocytes, the present investigation deals with the weights of these organs, the volumes of red and white splenic pulps and the plasma cell content of the spleen in guinea-pigs that have been subjected to subtotal thymectomy or sham-operation. Immunized as well as non-immunized animals are used, and these are examined 7 days after the operation. Thus the

organism is not given much time to compensate for the loss of thymus tissue. Guinea-pigs have been chosen for the experiments, since subtotal thymectomy does not stress them unduly, which is of importance when the animals are examined so soon after the operation.

Material and methods

72 male guinea-pigs, about 3 weeks old and weighing 138–214 g, were used. They were of the same strain as the animals in the previous investigations and were kept on the same diet. Half the animals were immunized with formalin-killed *S. typhi* (strain I.S. 57) in the same way and using the same doses as in the experiment described in the previous Chapter. The remaining animals were given equal volumes of physiological saline instead.

On the 19th day after the subcutaneous injection of bacteria or saline subtotal thymectomy or sham-operation was carried out. The subtotal ectomy took place in the way described in Chapter II. The two large thymus lobes were excised, cleaned from fat and adjacent tissue and weighed, while the lesser aberrant lobes were left *in situ*. According to *Gyllensten* (1953), this means that in newborn animals as a rule more than 95% of the thymus tissue is extirpated. The sham-operation was carried out as follows. After the incision the large thymus lobes were mobilized in the same way as in the ectomy, but no vessels were cut off. The subtotal ectomy as well as the sham-operation took 2–3 minutes. Both were carried out under sterile precautions. After the operation the booster dose or the saline was injected into the deep jugular vein. None of the animals seemed at all affected by either the immunization or the operation.

The total animal material comprised 4 equally large groups. The first was thymus-resected and received saline. The second was sham-operated and received saline. The third was thymus-resected and was given antigen. The fourth group, finally, was sham-operated and received antigen. In order to avoid possible disturbing influences of seasonal variations, temporary infections among the animals and other factors, the experiments were run simultaneously in all groups. The operations were carried out about the same time of day, in order to avoid possible differences due to diurnal changes. To eliminate great variations in weight between the groups the animals were ranked according to their weight and so placed that each group contained all types from the heaviest to the lightest. At the beginning of the investigation the mean body weight was:

First group 170.2 ± 3.9 (standard error) g,
Second group 170.4 ± 4.2 g,
Third group 171.3 ± 4.4 g,
Fourth group 171.7 ± 3.9 g.

The animals were weighed and killed 7 days after the operation, i.e. on the 26th day. The wound was opened under ether anaesthesia and about 3 cc blood taken from the deep jugular veins of all the animals, after which they were killed by a blow on the neck. The spleen, the large mesenteric lymph node situated at the main stems of the mesenteric vessels and the deep cervical lymph nodes of all animals and the two main lobes of the thymus of the sham-operated ones were dissected out, cleaned from fat and adjacent tissue and weighed. The nomenclature of the lymph nodes is according to *Glimstedt* (1936). No visible hyperplasia of the remaining thymus tissue could be detected in the resected animals.

The spleens were fixed in a mixture of alcohol, formalin and acetic acid according to *Serra* (1946). They were embedded in paraffin and cut in transverse sections 15 and 5 μ thick. The 15 μ sections, which were used to determine the volumes of the red and white pulps, were stained with Harris' haematoxylin and eosin, while the 5 μ sections were stained according to Brachet's modification of Unna-Pappenheim's methyl green-pyronin technique (*Pearse* 1960).

A method described by *Glimstedt* (1936), *Hammar* (1938) and *Gyllensten* (1953) and others was used to determine the volumes of the red and white splenic pulps. The sections were projected onto a paper by means of an apparatus that magnified about 14 times. The contours of the section and the outline of the white pulp against the red pulp were drawn, the perifollicular zone being regarded as belonging to the white pulp and the capsule and trabeculae to the red one. The vessels outside the trabeculae were considered to belong to the pulp in which they were situated. The drawings were cut out and the weights of the paper shapes representing the red and white pulps determined. These weights were used to find the volumes of the pulps in per cent of the total splenic volume. Every 36th section of 68 spleens was drawn and cut out. The number of sections examined in each spleen varied from 19 to 42, the mean being 27.

In order to get an idea of the random error when every 36th section was used, 12 such determinations were made on one spleen in each of the 4 groups. Thus every 3rd section was used in these 4 spleens and each determination comprised an examination of 25–31 sections. In each individual spleen the same number of sections was used for every determination. The results are recorded in Table 5.

At the end of the investigation the mean body weights were:

Thymus-resected and non-immunized animals	337.5 ± 9.4 g,
Sham-operated and non-immunized animals	339.3 ± 9.9 g,
Thymus-resected and immunized animals	327.0 ± 7.2 g,
Sham-operated and immunized animals	312.2 ± 9.7 g.

In the same 4 groups the mean thymus (the two lobes) weights were 489.1 ± 31.1, 486.9 ± 26.8, 539.6 ± 31.3 and 437.5 ± 37.6 mg respectively.

TABLE 5. *Volume of red splenic pulp in per cent of total splenic volume in the spleens where 12 determinations were made.*

Determination no.	Spleen of a thymus-resected and non-immunized animal	Spleen of a sham-operated and non-immunized animal	Spleen of a thymus-resected and immunized animal	Spleen of a sham-operated and immunized animal
1	81.9	85.6	84.0	90.4
2	81.7	85.6	83.7	90.6
3	81.4	85.4	83.9	90.4
4	82.4	85.8	83.9	90.4
5	82.6	85.4	83.6	90.3
6	82.4	84.9	83.5	90.9
7	82.6	85.8	84.6	90.7
8	83.3	85.4	83.1	90.3
9	81.8	85.6	83.6	90.2
10	82.0	85.7	83.9	90.9
11	82.3	85.7	84.3	90.2
12	81.7	85.8	84.2	90.8
Mean and standard deviation	82.2 ± 0.5	85.6 ± 0.3	83.9 ± 0.4	90.5 ± 0.3

In this investigation the reliability of the technique of immunization was also tested. (These tests were carried out by Ass. Prof. G. Laurell, Institute of Bacteriology, Uppsala.) The antibody content was determined in serum from 14 animals, half of which were thymus-resected, the method used being the same as that described in the previous Chapter. 1 animal (resected) had the titre of 1/20480, 3 (sham-operated) 1/10240, 4 (2 resected, 2 sham-operated) 1/5120, 3 (2 resected, 1 sham-operated) 1/2560 and 3 (2 resected, 1 sham-operated) 1/1280.

RESULTS

Tables 6 and 7 show the results of the weight determinations. The figures represent the means and their standard errors. If the weights of the organs, absolute as well as relative (weight of organ in mg per 100 g ultimate body weight), of the resected animals are compared with those of the sham-operated ones, no statistically significant differences are obtained either for the immunized or non-immunized animals ($P > 0.02$). The same is the case when the volumes of the splenic pulps expressed in per cent of the total splenic volume are compared in a similar way. For the animals

TABLE 6. *Mean weights of the organs in mg.*

Organ	Thymus-resected and non-immunized animals	Sham-operated and non-immunized animals	Thymus-resected and immunized animals	Sham-operated and immunized animals
Spleen	559.0±33.2	479.8±26.9	644.6±62.4	651.7±74.6
Mesenteric lymph node	292.1±18.5	314.1±19.9	305.3±15.1	340.1±27.8
Cervical lymph nodes	142.1±10.5	175.7±18.6	158.1±10.8	151.3±13.5

TABLE 7. *Mean relative weights of the organs (weights of the organs in mg per 100 g ultimate body weight).*

Organ	Thymus-resected and non-immunized animals	Sham-operated and non-immunized animals	Thymus-resected and immunized animals	Sham-operated and immunized animals
Spleen	163.8±7.4	141.6±5.9	196.6±18.1	212.2±27.4
Mesenteric lymph node	87.7±6.1	92.3±4.7	93.8±4.6	110.1±10.1
Cervical lymph nodes	42.1±3.5	52.8±5.8	48.5±3.2	50.2±5.5

that had received saline the mean percentage of red pulp was 85.5±0.5 (standard error) in the thymus-resected group and 84.1±0.8 in the sham-operated group. For the immunized animals the corresponding values were 87.3±0.9 and 85.2±1.2% respectively. According to the method used the remainder of the spleen consists of white pulp.

As a rule the plasma cell content of the spleens of the immunized animals was higher than that of the non-immunized ones. No certain differences could, however, be observed between the thymus-resected and sham-operated animals in both these groups.

Discussion

It may be asked to what extent the results obtained may agree with the assumption concerning a migration of lymphocytes from

the thymus to the spleen and lymph nodes under physiological conditions. All transfusion experiments with thymus lymphocytes indicate that these cells accumulate in the spleen to a much greater extent than in the lymph nodes, to which only a small number of cells can be traced. One would therefore hardly expect subtotal thymectomy to cause a decrease in the weights of the lymph nodes. It is also possible that the number of thymus lymphocytes migrating to the spleen is so small as to leave the weight of that organ apparently unaffected. The existence of a lymphocyte migration at least from the thymus to the spleen does in any case appear very probable considering the lively production of lymphocytes in the thymus and the results of the transfusion investigations.

Another possible explanation for the absence of any differences between the weights of the organs of resected and sham-operated animals is that in the former spleen and lymph nodes have had time to develop a compensatory hyperplasia. *Gyllensten* (1953) carried out subtotal thymectomy on newborn guinea-pigs and 14 days later found that the lymph nodes of these animals weighed more than the same organs in sham-operated controls. After 28 days, however, no differences were found between the resected and sham-operated animals. Not at any time were any certain differences obtained when comparing the spleen weights. The change in the weight of the lymph nodes after 14 days was thought to be due to a compensatory hyperplasia, an assumption supported by the results of a later investigation in which the uptake of P^{32} in the lymphatic tissue of guinea-pigs was studied after subtotal thymectomy (*Gyllensten & Ringertz* 1954).

Schooley & Kelly's (1958) research into the weight of lymph nodes after thymectomy showed that in thymectomized rats these organs weighed less than in sham-operated controls. The operations were done when the animals were 6 days old and the organs examined after 54 days. Apart from determining the weights the authors studied the DNA synthesis by means of P^{32}. They found no differences between thymecotmized and sham-operated animals concerning the specific activities of DNA in the lymph nodes and spleen.

Thymectomy on rats was also carried out by *Bierring* (1960), who found no differences between thymectomized and sham-operated animals regarding the weights of spleen and lymph nodes. He also studied the mitotic frequency in these organs but could

not observe any differences there either. The operations were performed on rats weighing 70 g and the animals were killed 65 days later.

Fichtelius & Diderholm (1959 *a*) performed thymus-resection or sham-operation on 1-month-old guinea-pigs which were then injected with P^{32} in the form of orthophosphate. Six hours and 4 and 8 days later the spleens were examined by means of apposition autoradiography, which showed that the radioactivity in the red pulp of the resected animals on the two later occasions was less than in the controls. It was suggested that this could be due to a decrease in the supply of labelled lymphocytes to the spleen after the resection. No weight determinations were made. Later *Fichtelius, Diderholm & Stillström* (1960) carried out an almost identical investigation except that the radioactivity of certain phosphorus fractions was measured, a G-M counter being used. In this investigation, which also included animals that had received antigen, no differences were found between thymus-resected and sham-operated animals regarding the specific activities in the spleen and lymph nodes. As to weight differences between these organs, the results were equally negative. All determinations were made 4 days after the operation.

Hammar (1938) found that in rabbits immunized with *S. paratyphi* B thymectomy caused a decrease in the weights of the spleen and lymph nodes as compared to controls that had not been sham-operated. The red splenic pulp was reduced, whereas the white pulp remained unchanged. The animals were ectomized at the age of 3 months and killed 2 months later.

The results of the present investigation thus diverge from those reported by *Gyllensten* (1953), *Schooley & Kelly* (1958) and *Hammar* (1938). To what extent this is due to such factors as differences in the type of animal used or in the point of time after operation chosen to examine the organs it is difficult to make out. In *Hammar's* investigation a stress effect upon the animals in connection with the ectomy may have affected the results.

An explanation for the findings of *Schooley & Kelly* (1958) and *Hammar* (1938) may be that fewer lymphocytes reach the organs in question in ectomized animals than in controls. It is also possible that the ectomy causes an increase in the release of cells from these organs. It may be that some of these released cells are directed to other parts to replace the thymus lymphocytes. The results of the

research with guinea-pigs and *Bierring's* (1960) investigation do not exclude the possibility that quite a number of thymus lymphocytes are transported to the spleen and lymph nodes, however, since some compensatory mechanism may come into play after resection. Thus in the case of guinea-pigs subjected to subtotal thymectomy the remaining thymus tissue might produce and release a greater number of lymphocytes than under physiological conditions.

It must emphasized that the production of lymphocytes is only one of several functions ascribed to the thymus. The closer effect of thymectomy upon the spleen and lymph nodes must be complicated. Other factors than possible changes in the migration of lymphocytes to or from these organs or in the intensity of cell formation within them may affect their weights after thymectomy.

The plasma cell content of the spleens of resected and sham-operated animals appeared more or less the same, and thus the present investigation adds nothing to support *Fichtelius'* (1953, 1957 *c* and 1960) hypothesis that thymus lymphocytes are able to turn into plasma cells.

Summary

Immunized and non-immunized guinea-pigs were used to study the influence of subtotal thymectomy on the weights of spleen and lymph nodes, on the volumes of red and white splenic pulps and on the plasma cell content of the spleen.

The weights of organs from thymus-resected and sham-operated animals were compared with each other, first in non-immunized animals, then in animals that had been immunized with *S. typhi*. No statistically significant differences were found either concerning the absolute weights or when these had been related to the body weights of the animals. A corresponding comparison between the volumes of the splenic pulps calculated in per cent of the total splenic volume and the plasma cell content of the spleen showed a similar absence of differences.

The question discussed in the first place is to what extent the results obtained may agree with the assumption concerning a migration of lymphocytes from the thymus to the spleen and lymph nodes under physiological conditions. The results are also compared with observations by other authors concerning changes in the weights of these organs after thymectomy.

CHAPTER VI

The Number and Distribution of Labelled Cells in Spleen and Thymus of Rats at Different Intervals after the Administration of H^3-Thymidine

In order to study the fate of lymphocytes after leaving the blood, transfusion experiments with labelled or unlabelled lymphocytes from the thymus, lymph nodes, lymph ducts and blood have been carried out (references in Chapter VII). This type of experiment suffers from certain drawbacks when it comes to investigating the fate of the lymphocytes under physiological conditions. Some of these were pointed out in Chapter V, where another method of studying this problem was described. No conclusions could be drawn from that investigation, however.

In order to back up the assumption based on the results of earlier transfusion investigations that lymphocytes in the blood accumulate in the spleen, *Diderholm & Fichtelius* (1959 *c*) studied the location of P^{32} in the spleen of young rats at different intervals after an injection of P^{32}-orthophosphate by means of apposition autoradiography. It was shown that after 6 hours the blackening of the film mainly appeared over the red pulp and over a small spot at the centre of some follicles. After 4 and 8 days, on the other hand, the white pulp gave rise to heavier blackening than the red. One of many possible interpretations of the results was that the blackening over the red pulp after 6 hours was due to immigrant lymphocytes from the thymus (and perhaps also lymph nodes) possessing a rapid turnover of DNA and reaching the spleen before that organ's own lymphocytes had had time to be labelled.

The difficulties encountered in interpreting the results of the above investigation were at least partly due to the facts that P^{32}-phosphate is not incorporated into DNA alone, that the P^{32}-phosphate content of the organs is rather high even several days after the injection and that apposition autoradiography does not permit observations at the cellular level. If H^3-thymidine and stripping autoradiography are used, the conditions are entirely dif-

ferent. It is generally assumed that thymidine is incorporated specifically into DNA and that it is available for the DNA synthesis only for a short period after the injection. The distribution of labelled cells in the spleen a short time after the injection with H^3-thymidine yields information concerning the regions where the DNA-synthesis is taking place. At a later point many of the labelled cells present may be such as have immigrated from other organs.

In the present investigation, the aim of which is to find support for the theory that lymphocytes from the blood accumulate in the spleen under physiological conditions, spleen and thymus are studied by means of stripping film autoradiography at different intervals after the administration of H^3-thymidine. Moreover the results are compared to those obtained with P^{32} and apposition autoradiography.

Material and methods

33 male rats (Sprague-Dawley strain), about 21 days old and weighing 49–63 g, were used. All the animals, which were kept on a standardized diet, received intraperitoneally 0.5 μC H^3-thymidine[1] per g body weight. The thymidine had a specific activity of 2.7 C per mmole and had been dissolved in physiological saline so that 0.5 cc contained 25 μC.

The animals were killed by means of ether inhalation: 11 animals after 1 hour, 11 after 6 hours and 11 after 4 days. Spleen and thymus were dissected out, fixed in neutral 10% formalin and embedded in paraffin. Sections 6 μ thick were cut.

The autoradiographic technique and the type of film were the same used in the previous investigations (see Chapter II). The exposure time was 40 days, the methods of developing, fixing and rinsing, the solutions used and the times taken by these procedures were the same as in Chapter II. Some of the sections were stained with Ehrlich's haematoxylin, others with methyl green–pyronin according to Unna-Pappenheim.

At least 4 grains were required over a cell for it to be considered labelled. The examination of the spleen was more detailed than that of the thymus. The number of labelled cells was counted in 12 sections of every spleen. From each section 20 fields of vision were selected, 10 in the red pulp and 10 in the white. One field of vision corresponded to an area of about 7.8×10^3 μ^2 of the section. In the white pulp follicles without germinal centre were chosen, while in the red pulp the choice was limited to regions not bordering on the white pulp. In every spleen 1672–7071 cells (on the average 3367 cells) were counted. Apart from

[1] Supplied by Schwartz Laboratories Inc., New York.

the determination of the number of labelled cells per field of vision, their location within the pulps and their degree of labelling were also examined. The connective tissue was not taken into account. The labelled cells were moreover classified as to types.

Results

The results are given in Table 8, where the figures represent the means and their standard errors. As seen from the Table, the mean number of labelled cells per field of vision in the red pulp was 25.1 ± 2.0 one hour after the injection, the corresponding value for the white pulp being 5.5 ± 0.3. About 20% of the total number of nucleated cells in the red pulp was labelled, but less than 5% in the white. Within both pulps the cells were usually diffusely spread, though now and then groups of labelled cells were observed in the red pulp. No certain difference in the degree of labelling was found between the cells in the red and in the white pulp. The average number of grains over each cell was about 15. Plate 9 shows an autoradiogram of the spleen of an animal killed after 1 hour.

Six hours after the injection the mean number of labelled cells per field of vision in the red pulp was 39.3 ± 3.4 and in the white pulp 6.0 ± 0.3. Comparing the values from animals killed after 1 hour a statistically significant increase appeared in the red pulp ($0.01 > P > 0.001$), while no certain difference in the number of labelled cells was observed in the white. As was the case after 1 hour, the cells were usually diffusely spread over both pulps. Now and then groups of cells were observed in the red pulp, however. Over the majority of labelled cells in this pulp the number of grains was lower than was the case with those in the white pulp. Apart from rather weakly labelled cells, there also were a few as strongly labelled as those in the white pulp, their degree of labelling being about the same as that of the cells in the spleen of animals killed after 1 hour. Plate 10 shows an autoradiogram of the spleen of an animal killed 6 hours after the injection.

The labelling shown by the autoradiograms of the spleen of animals killed 4 days after the injection differed completely from what was described for the animals killed after 1 and 6 hours. The white pulp contained more labelled cells per field of vision than the red; the mean numbers were 9.8 ± 0.4 and 6.9 ± 0.3 respectively. A comparison with corresponding values after 6 hours shows

TABLE 8. *Mean number and mean ratio of labelled cells per field of vision in red and white splenic pulps.*

Time after injection	Mean number of labelled cells per field of vision		Mean ratio of labelled cells per field of vision in red to white pulp
	Red pulp	White pulp	
1 hour	25.1±2.0	5.5±0.3	4.5±0.2
6 hours	39.3±3.4	6.0±0.3	6.4±0.3
4 days	6.9±0.3	9.8±0.4	0.71±0.02

a statistically significant decrease of the number of labelled cells in the red pulp and a statistically significant increase of the cells in the white ($P < 0.001$ in both cases). Within both pulps the cells seemed evenly distributed. The degree of labelling of the cells had decreased considerably compared with the spleens from the animals in the first two groups. This was especially true of the cells in the red pulp. Plate 11 shows an autoradiogram of the spleen of an animal killed after 4 days.

Determinations of the ratio between the number of labelled cells per field of vision in the red pulp and that in the white pulp were made for all the spleens and the means of these are also given in Table 8. As is seen from the Table, the ratio after 6 hours is higher than after 1 hour, the difference being statistically significant ($P < 0.001$). After 4 days the ratio is lower than after 1 and 6 hours, and these differences, too, are statistically significant ($P < 0.001$ in both cases).

In both pulps of all the spleens the majority of labelled cells were classified as lymphocytes of different sizes. Further, there were quite a number of labelled reticular cells, while rather few plasma cells and granulocytes were found to be labelled. A large number of labelled cells, most of them immature types, could not be identified. In the spleens of the group killed after four days proportionally more labelled lymphocytes were observed than in the other animals, while no certain difference was noted between the animals killed after 1 hour and those killed after 6 hours.

In the thymus labelled cells were found in the cortex as well as the medulla. The number of labelled cells in the cortex of animals killed after 1 hour was on the average about 20 per field of vision, which was about 3–4 times as many labelled cells as in

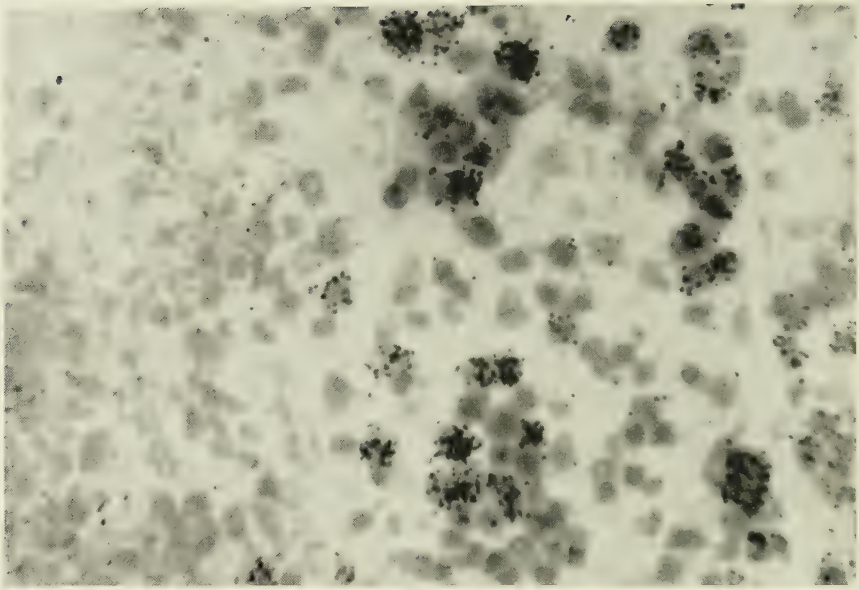

Plate 9. Autoradiogram of the spleen from an animal killed one hour after the administration of H^3-thymidine. The red pulp (to the right) contains a fair number of labelled cells, many of them strongly labelled. (Haematoxylin, × 720.)

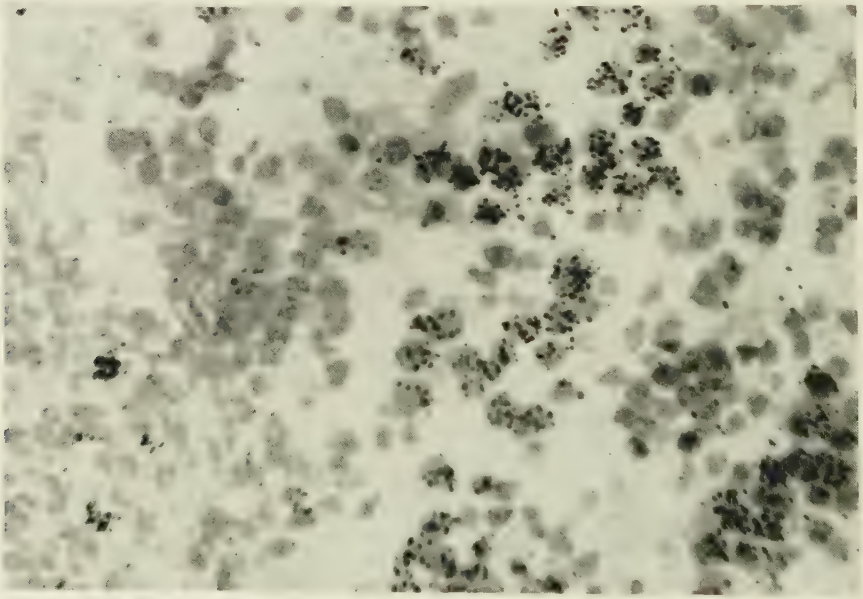

Plate 10. Autoradiogram of the spleen from an animal killed six hours after the administration of H^3-thymidine. At this stage the labelled cell content of the red pulp (to the right) is very high and most of the cells are moderately labelled.
(Haematoxylin, × 720.)

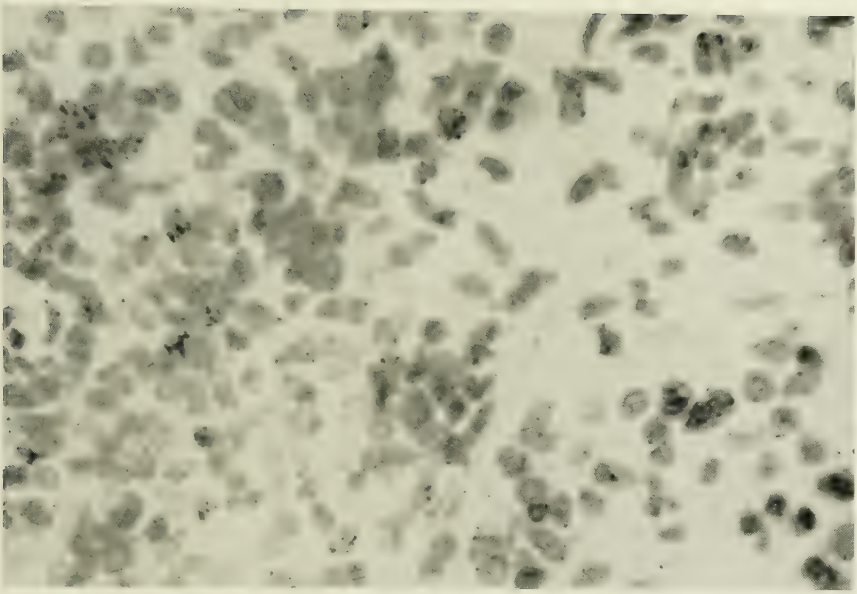

Plate 11. Autoradiogram of the spleen from an animal killed four days after the administration of H^3-thymidine. Of the few, weakly labelled cells observed many are localized in the white pulp (to the left). (Haematoxylin, × 720.)

the medulla per field of vision and about 3–7 % of all the cells in the cortex. In the medulla the labelled cells accounted for about 5–10 % of all cells. As a rule the cells in the cortex were rather more weakly labelled than those in the medulla. Altogether the thymus cells were labelled rather less than those in the spleen of the same animal. A comparison between the animals killed after 1 hour and those killed after 6 hours disclosed no certain differences concerning the number of labelled cells and their distribution. Concerning the degree of labelling, the cells after 6 hours were possibly somewhat more weakly labelled than those after 1 hour. After 4 days the number of labelled cells as well as their degree of labelling had decreased considerably. In the cortex as well as the medulla only a few weakly labelled cells were found. More than 90 % of the labelled cells in the thymus were classified as lymphocytes, the rest being mainly reticular cells. This was the case for all three groups.

The number of background grains was rather low in all the autoradiograms, about 3–5 grains per 1000 μ^2.

Discussion

The essential premises involved in the use of H^3-thymidine for labelling DNA have been laid down by *Hughes et al.* (1958), *Cronkite, Bond, Fliedner, Rubini* (1959) and *MacDonald & Mallory* (1959). They also form the basis of the present investigation. Thymidine is incorporated specifically into DNA and only during synthesis preparatory to cell division, and it is available for a short period after injection. The turnover of the labelled DNA is either the result of mitosis or cell death. During mitosis the labelled DNA is distributed to daughter cells and in a non-dividing cell formed from a labelled cell the labelled DNA is stable for the life of the cell.

The labelled cells in the spleen and thymus can be: (1) cells that have incorporated the isotope in these organs, (2) cells that have incorporated the isotope in other organs and have then migrated to the organs in question and (3) cells that have been formed by mitosis of the cells named under (1) and (2).

A comparison between the autoradiograms of the spleens taken 1 and 6 hours after the injection showed at the latter time a larger number of labelled cells per field of vision in the red pulp as well as a higher ratio between the numbers of labelled cells in the pulps. A probable explanation for this may be that labelled lymphocytes have migrated to the spleen, where, as suggested by transfusion experiments with thymus and lymph node lymphocytes in guinea-pigs (*Diderholm & Fichtelius* 1959 *a* and *b*, Chapters II and III) and thymus lymphocytes in rats (*Diderholm*, unpublished), they are recovered in greater numbers per field of vision in the red than in the white pulp. It can be supposed that practically all H^3-thymidine administered has been incorporated into DNA 1 hour after the injection (*Hughes et al.* 1958, *Cronkite et al.* 1959, *MacDonald & Mallory* 1959 and *Nygaard & Potter* 1959). Four days after the injection the mean number of labelled cells per field of vision in the red pulp and the calculated ratio were lower than after 1 as well as 6 hours. The reason for this may be that lymphocytes migrating to the spleen some time after the injection are not labelled and that the isotope content of many cells, being the result of mitosis, is too small to be registered by the film.

If labelled lymphocytes have migrated to the spleen from 1 to 6 hours after the injection, the question arises whether the total

radioactivity in that organ or at least in its red pulp ought not to be higher at the later time. According to the autoradiograms this was probably not the case. Though the number of labelled cells in the red pulp did increase, most of the cells were rather weakly labelled, and as a result the radioactivity appeared to remain the same. This does not contradict the assumption concerning a migration of labelled lymphocytes to the spleen between 1 and 6 hours, since it may be possible that at the latter time many of the cells, being the result of mitosis, may contain so little isotope that the film has not registered them.

A comparison betwen autoradiograms of the thymus of animals killed after 1 and 6 hours revealed no certain differences concerning the number of labelled cells and their distribution. Possibly the degree of labelling of the cells was somewhat lower at the latter time. Thus no evidence was found to indicate that lymphocytes left the thymus to any great extent during the first 6 hours of the experiment. This may, however, have been the case all the same, since a migration of labelled cells to the thymus can have taken place. On the other hand the changes in the labelling of the spleen at the different intervals can have been produced by lymphocytes formed in other organs than thymus. After 4 days the thymus contained only weakly labelled cells. It is impossible to say what this is due to. It may be an effect of a migration of labelled cells from the thymus and of a dilution of the isotope by repeated cell divisions, making autoradiographic registration impossible.

However, there are other possible explanations for the changes in the labelling of the splenic pulps at the different intervals. It may be, for instance, that the labelled cells in the red pulp have a greater tendency to mitosis. Four days after the injection many of the cells may have divided several times, in which case the isotope content in the cells has become so small that it is no longer registered by the film. Other differences between the cells in the two pulps may also have had an influence. Thus it is possible that the results have been affected by such factors as differences in the radiosensitivity of the cells, release of isotope from dead cells and differences in the possible incorporation of the released isotope into other cells.

On the whole, the results of the present investigation are similar to those obtained with P^{32} and apposition autoradiography (*Diderholm & Fichtelius* 1959 *c*). As previously mentioned, the blackening

of the film 6 hours after the injection occurred mainly over the red pulp and over the centre of some follicles. After 4 and 8 days, however, the blackening over the white pulp was heavier than over the red. At these times the radioactivity within the white pulp appeared evenly distributed. Since the investigation with H^3-thymidine showed no accumulations of labelled cells in the centre of the follicles, it seems very likely that the blackening encountered there after 6 hours was caused by P^{32} not bound to DNA.

Summary

The number and distribution of labelled cells in the spleen and thymus of young rats was studied by means of autoradiography at different intervals after the administration of H^3-thymidine. One hour after the injection the number of labelled cells per field of vision was higher in the red than in the white splenic pulp. After 6 hours the number of labelled cells per field of vision in the red pulp had increased, while no certain difference concerning their number could be observed in the white. Four days after the injection the situation had changed completely: the number of labelled cells per field of vision had decreased considerably and the white pulp contained more labelled cells per field of vision than the red.

The differences in the labelling of both pulps at the different intervals may be due to a migration of lymphocytes to the spleen, where, as suggested by transfusion experiments, they have a greater tendency to accumulate in the red pulp than in the white. Other explanations can be suggested, and some of these, as well as the behaviour of the thymus lymphocytes during the course of the experiment, are discussed.

CHAPTER VII

General Discussion

A. DISCUSSION OF METHODS

Transfusion of labelled lymphocytes as a method of studying the fate of lymphocytes after leaving the blood

As has already been pointed out in the Introduction, the problem of the function of lymphocytes must be intimately connected with the fate of these cells after leaving the blood. In order to investigate this matter the method of transfusing labelled as well as unlabelled lymphocytes has been used in recent years. However, all transfusion investigations have certain drawbacks when it comes to studying the behaviour of the cells under physiological conditions. The cells may be damaged during the preparation and injection of the cell suspension or by the labelling process, whether fluorescent dyes or radioactive isotopes are used. Usually the quantity of cells injected over a short period is too large for an adequate resemblance to physiological conditions. Moreover, when lymphocyte suspensions made of thymus or lymph nodes are transfused, it can be assumed that they contain many cells that under physiological circumstances might never reach the blood at all or which are too immature to be released to the blood at the particular time the transfusion is made.

The question arises to what extent the above or other factors have influenced the results obtained so that the physiological behaviour of lymphocytes is not reflected. Apart from lymphocytes the cell suspensions contained labelled reticular cells, which are generally believed to be able to turn into lymphocytes. In the organs examined labelled reticular cells were also found, but apparently only a small proportion of the number transfused. Since the labelled cells were sometimes difficult to classify, it was impossible to compare the decrease in the number of labelled reticular cells with

that in the number of labelled lymphocytes after transfusion. There is a chance that some labelled reticular cells subsequently developed into lymphocytes, perhaps after first having been transported to some organ. This possibility represents a source of error, since the migration of lymphocytes is the object of study. Most probably only a small minority of all the lymphocytes registered have developed from labelled reticular cells, however.

In the Introduction it was emphasized that we do not know what proportion of the lymphocytes in the thymus reach the blood, but probably a rather large part do. In the transfusion experiments presented here it can be assumed that some of the cells registered in the organs are such as would, under physiological conditions, never have reached the blood at all or such as were too immature to be released to the blood. What proportion of the recovered cells these comprise and whether they behave differently from cells normally released to the blood it is impossible to say. However, at the time of the transfusion the majority of the lymphocytes labelled with S^{35} were small, which indicates that most of them were ready to be released to the blood. The facts that lymphocytes in the blood can incorporate thymidine *in vitro* (*Schooley, Bryant & Kelly* 1959) and that most of the H^3-labelled lymphocytes in the investigations were medium-sized when the transfusion took place make it very likely that in this case, too, a fairly large proportion may be considered sufficiently mature.

In investigations into the destruction of red cells *Jandle, Jones & Castle* (1957) found that the spleen, liver and lung function as passive filters, the smaller agglutinates being caught mainly in the spleen, while the larger are taken up by the liver and lung. This suggests that perhaps some of the transfused lymphocytes were aggregated and were passively filtered out into the organs. However, when the cell suspensions were microscopically examined cell aggregations were rarely found, and, moreover, the labelled cells in the sections were usually seen singly.

It is conceivable that the cells killed according to Schrek's test and perhaps other, not fatally injured cells as well are taken up by phagocytes. The isotope may be retained in the phagocytes, which later may turn into lymphocytes. This theory is supported by an investigation by *Trowell* (1957) showing that pyknotic lymphocytes are phagocytosed and that the phagocytes then change into large lymphocytes. Actually in the present research not a

single labelled phagocyte was observed with any certainty, but it is possible that such cells were present earlier and at the time for killing the animals they had been developed into labelled lymphocytes. However, the probability for this seems slight in the light of the control experiments, in which no labelled cells were found.

Stress in connection with the transfusion and the fact that rather a large number of cells was transfused over a short period are other possible factors affecting the results. However, according to *Osogoe* (1950), the cells first gather in the capillaries of the lung, whence after some time they migrate to the spleen and liver. This suggests that the rate at which the lymphocytes are given off from the lung is more physiological than that at which they are transfused. *Gowans* (1957, 1959 *a* and *b*) has transfused lymphocytes at approximately the rate at which the cells are believed to enter the blood. These investigations were criticized by *Yoffey* (1960), however, principally on the ground that the animals were considerably affected by stress. In the present investigations the stress the animals were exposed to must be considered to be rather slight.

It has been difficult to determine by means of the autoradiograms to what extent the transfused lymphocytes had left the blood in the organs examined. A few were recovered in the capillaries, and in the spleen some of them appeared to be lodged in the sinusoids. The recovery of transfused lymphocytes in certain organs may perhaps be partly connected with the vascularity of these.

The above discussion shows that there are many factors influencing the question to what extent the transfusion investigations can give a true picture of the physiological behaviour of lymphocytes. To what degree these factors have influenced the distribution of the transfused cells must naturally remain a matter of conjecture. Most probably, however, a fairly large part of the transfused cells have behaved as thymus lymphocytes would under physiological conditions.

Even if the transfusion experiments are not altogether unexceptionable concerning the study of the migration of lymphocytes, they give valuable information about the capacity of lymphocytes to turn into other cells. It is true that cells injured by radioactive isotope or other factors may have lost their possible property of transformation, but in the present investigations the majority of the labelled cells transfused may be considered intact.

Choice of thymus lymphocytes for transfusion

The quickest way of obtaining large numbers of lymphocytes is to take them from the thymus of young animals. Guinea-pigs are especially suitable on account of the position of most of the thymus tissue subcutaneously on the neck. A large part of the organ can thus be easily excised without causing the animals undue stress, which is very important when autologous transfusions are to be performed. The advantages of this type of transfusion become apparent when the following points are discussed. In the investigation reported in Chapter IV some of the animals were killed $5\,^1/_3$ days after the transfusion. If homologous instead of autologous transfusions had been carried out, it is probable that only a few or no transfused cells at all would have been recovered, since the recipients could be expected to form antibodies against the homologous cells. Had isologous transfusions been made it would probably have been possible to trace the lymphocytes after $5\,^1/_3$ days. However, as donors and recipients may be differently immunized with antigens entering through the digestive and respiratory tracts or in some other way, the distribution of the transfused lymphocytes in the recipients would probably not be the same as in the animals where they originated.

As pointed out previously in this Chapter, the use of thymus cells for transfusion has certain drawbacks, however. Cells that would normally never reach the blood or are too immature to be released may be registered. The ideal would be to use lymphocytes from the thoracic duct instead, but this would make autologous transfusions more difficult.

Choice of H^3-thymidine and S^{35}-methionine for labelling lymphocytes for transfusion

The methods of labelling lymphocytes for transfusion experiments have been to use radioactive isotopes or fluorescent dyes (references in Chapter VII). Labelling with fluorescent dyes seems to have certain disadvantages. The presence of autofluorescence in the tissues complicates the interpretations. Further, the dye fades from the transfused cells only after a few hours, and there is a risk that it is taken up by other cells, which are then labelled instead. *Keohane & Metcalf* (1958) point out that *Farr's* (1951) observations of a transformation of transfused labelled lymphocytes into

myelocytes may be erroneous, as it is possible that the myelocytes simply took up the dye released from the transfused cells.

Of recent years it has become more and more common to use radioactive isotopes when labelling lymphocytes. For locating the cells within the organs the autoradiographic method has been found most convenient. In order to be able to trace each individual transfused cell the autoradiograms must have a high resolving power, which can be reached by using stripping film autoradiography and labelling the cells with an isotope emitting low-energy beta particles. H^3 and S^{35}, emitting beta particles with a maximum energy of about 0.018 and 0.167 MeV respectively (*Kamen* 1951), have proved suitable. In the present investigations the H^3-labelled cells could be traced several days after transfusion, while those labelled with S^{35} were recovered after 8 hours. Even the last-mentioned period is considerably longer than those during which *Hagerman* (1954) and *Keohane & Metcalf* (1958) could trace lymphocytes labelled with fluorescent dye. The choice of H^3-thymidine and S^{35}-methionine has also been influenced by the fact that the cells could be labelled *in vitro*, making autologous transfusions possible. H^3-thymidine labels only a restricted number of lymphocytes, presumably mainly those cells synthesizing DNA, while S^{35}-methionine is incorporated by a greater number of cells. This has made it possible to study divergences between different types of lymphocytes.

Radiosensitivity of lymphocytes

Lymphocytes are generally recognized to be sensitive to ionizing radiation. H^3 administered in the form of thymidine is concentrated in areas believed to be very sensitive, actually in the chromosomal structure. It may be asked whether the isotope doses used in the present investigations were so high as to injure the cells.

Schrek's test showed no certain difference between the frequency of dead cells before and after the labelling. In all the investigations the smears and sections were examined for radioactive cell injury, but there were no definite indications that any such damage had occurred.

The problem of radiation injury by H^3-thymidine to the lymphocytes of rats has been investigated by *Cronkite, Bond, Fliedner & Killman* (1960), who used the Trowell technique to show that H^3-thymidine in doses of less than 1 μC per g body weight is relatively

innocuous. The radiation effects of H^3-thymidine on the morphology of spermatogonia in mice were studied by *Johnson & Cronkite* (1959). They showed that a dose of 5 μC per g body weight is comparable to 10 r gamma radiation per day and that doses less than 1 μC have no effect. Lymphocytes and spermatogonia have about the same degree of radiosensitivity (*Cronkite et al.*).

The evidence of the investigations quoted above, as well as the checks made in the present studies, strongly indicate that the doses used here have been kept below the limit at which they can cause morphological injury. However, the possibility cannot be neglected that injured cells were present in the organs before the animals were killed, but that these disappeared before they could be registered. Moreover, functional damage to cells containing radioactive isotope in many cases may not be detectable by the usual morphological criteria. The question concerning differences between the behaviour of labelled and unlabelled cells therefore cannot at present be answered.

Registration of labelled lymphocytes by means of stripping film autoradiography

The number of grains used in the present investigations as a measure of the degree of labelling of the cells does not depend on the isotope content of the cells alone. Many other factors may play a part, and cells with exactly the same isotope content may give different grain counts owing to the varying effect of these factors. Thus the grain count may be influenced by variations in sensitivity of the film, section thickness, distance of the film from the section, presence of certain substances in the section causing so-called chemical blackening, presence of ionizing radiation other than that from the injected isotope, temperature and humidity during the stripping procedure and the exposure and development of the film (*Boyd* 1955).

It may be asked how great the influence of these factors was on the present results. The relatively slight variations in the mean number of grains over the labelled lymphocytes (see Tables 1–4) and in the mean number of labelled lymphocytes per 100 fields of vision in the spleens in which 12,000 lymphocytes were counted (see Tables 1 and 3) suggest that it was negligible. To find support for this assumption the mean number of grains over every 3rd

labelled lymphocyte in one and the same section was calculated. Twenty-five sections were used of the spleen in which 12,000 S^{35}-labelled lymphocytes had been counted. In each section, each of which was covered with only one piece of film, the number of grains was determined over 160 lymphocytes. The mean value of the 25 determinations was calculated as 20.2 grains, the standard deviation as ± 1.1 and the coefficient of variation as $\pm 5.3\%$. Twenty-four sections were used of the spleen in which 12,000 H^3-labelled lymphocytes had been counted. Under each piece of film there were 3 sections, and in every section the number of grains was determined over 80 lymphocytes. The mean value of the 8 determinations was 14.3 grains, the standard deviation ± 0.7 and the coefficient of variation $\pm 4.7\%$. The values obtained strongly indicate that the effect of variations in film sensitivity, section thickness and the other factors cannot have influenced the results to any significant degree.

A final point that must be considered is that the number of grains over a cell probably only yields approximate information concerning its isotope content. It may be that the individual grains over cells rich in isotope are hit by more beta particles than those over rather weakly labelled ones. Moreover it is difficult to make an exact grain count over the most strongly labelled cells. The variations in the isotope content of the transfused lymphocytes in the different animals are therefore probably greater than shown by the mean grain counts (see Tables 2 and 4). All the same, the information given by the film concerning the isotope content of the cells is in all probability sufficiently accurate to justify the conclusions drawn in Chapters II and III about the nature of the lymphocytes registered.

B. DISCUSSION OF RESULTS

Comparison between results of the present and other transfusion investigations

The lymphocytes used in transfusion investigations have been of different types and the methods of labelling and tracing them have often varied. There have also been differences regarding the type of transfusion used—autologous, isologous, homologous or heterologous –the species of animal employed and the period allowed to elapse

before examination. The results of the various investigations can thus be expected to vary. For the sake of clarity they are here presented in tabular form, Table 9 giving a review of the earlier experiments in which lymphocyte suspensions made of thymus have been transfused, Table 10 listing those with suspensions made of lymph nodes and Table 11 those with lymphocytes from the lymph or blood. Not all transfusion investigations with lymphocytes have been included but only those principally concerned with the problems regarding the migration of lymphocytes to different organs and their ability to turn into other types of cells.

In the present investigations the transfused lymphocytes were recovered mainly in the spleen and liver. To a lesser extent they were traced to the mesenteric lymph node, lung and bone marrow. No cells were found in the intestinal wall, skin or blood. The Tables show that in most of the investigations the lymphocytes have been recovered in one or several of the five organs to which the H^3- and S^{35}-labelled lymphocytes were traced. In no less than four cases they are said to have migrated to the intestinal wall, namely in the investigations by *Farr* (1951), *Ambrus & Ambrus* (1959), *Shorter & Bollman* (1960) and *Everett, Rieke, Reinhardt & Yoffey* (1960). *Farr* and *Everett et al.* recovered the cells in Peyer's patches. *Fichtelius* (1959*a*) traced lymphocytes to the skin, and *Hagerman* (1954) presumably found transfused lymphocytes in skin sensitized to dinitrochlorobenzene. The differences between the results shown in the Tables are probably due to many factors, some of which are discussed below in connection with further comparison between the present and other results.

In the investigations in which unlabelled or P^{32}-labelled lymphocytes were transfused the cells were generally found in only a few of the organs examined, often in the spleen and liver. In those investigations where the individual labelled cells could be discovered, on the other hand, the cells were usually traced to several organs. The fact that in the present investigations (Chapters II and III) so many of the transfused lymphocytes were recovered in the spleen and liver strongly indicates that the number of unlabelled or P^{32}-labelled lymphocytes required in each organ for recording to become possible must be rather large.

The interval between the transfusion and the killing of the animals has varied in the investigations, a fact that has certainly contributed to the differences in the results. The Tables indicate

that when the animals had been killed after a short interval relatively more lymphocytes were recovered in the lung. *Weisberger et al.* (1951) showed that after 10 minutes the cells were found in the lung and spleen and possibly also in the kidney and liver. *Keohane & Metcalf* (1958) report that these results agree with their own, which refer to the distribution up to 2 hours after the transfusion. *Osogoe* (1950), stating no exact time, found a rather heavy accumulation of cells in the lung shortly after the transfusion. It appears from the Tables that where longer periods had been allowed to pass the lymphocytes were as a rule recovered mainly in the spleen and liver, while few or none were traced to the lung. *Osogoe* points out that a certain time must elapse before lymphocytes can be recovered in the particular regions of the liver and spleen, even when suspensions are injected directly into the portal circulation. He writes: "This fact suggests that the process is essentially no longer mechanical, but characteristic of the lymphocyte."

When studying the divergences in behaviour between different types of lymphocytes it is interesting to compare the investigations listed in the three Tables. Such a comparison is complicated by the fact that many of the factors that can be expected to influence the distribution of the transfused lymphocytes have varied. There are, however, some cases in which cells of different kinds have been transfused under rather similar experimental conditions. *Osogoe* and co-workers (1950, 1950 and 1954) arrived at the same results regardless of whether the cells were taken from the thymus or the lymph nodes. *Fichtelius* (1958 *a* and *b*), on the other hand, showed that transfused cells from the thymus and lymph nodes were distributed differently in the recipients. In his earlier investigation the thymus cells were traced to the liver and spleen 24 hours after the transfusion, while the lymph node cells were recovered in the liver and bone marrow and probably also in the lymph nodes and the lung. In his later work, in which the recipients were killed after 48 hours, similar results were obtained. By means of apposition autoradiography *Diderholm & Fichtelius* (1959 *b*) showed that transfused thymus cells had a greater tendency to accumulate in the spleen than cells from lymph nodes and that, in contrast to thymus cells, the lymph node node cells were also traced to the mesenteric lymph node.

According to investigations by *Fichtelius* (1953, 1957 *a* and

TABLE 9. *Review of experiments in which lymphocyte suspensions made of thymus were transfused.*

Author	Type of transfusion. Experimental animals	Method of labelling the lymphocytes	Method of identifying the lymphocytes after transfusion	Interval after transfusion. Location of the lymphocytes
Osogoe & Hitachi 1950	Homologous. Rabbits	No label	Examination of histological sections	72 hours. Liver (interlobular spaces) and spleen (perifollicular regions)
Fichtelius 1953	Homologous and heterologous. Rats. Heterologous cells from rabbits	P^{32}-phosphate *in vivo*	Determination of P^{32}-DNA in G-M counter	24 hours. Homologous cells in spleen and liver. Heterologous cells in liver
Hagerman 1954	Homologous. Guinea-pigs sensitized with dinitrochlorobenzene	Fluorescent dye *in vitro*	Fluorescence microscopy	1 and 2 hours. Cells very probably in skin
Karasawa & Osogoe 1954	Homologous. Rabbits	No label	Examination of histological sections	48–72 hours. Liver (interlobular spaces) and spleen (perifollicular regions)
Fichtelius 1957 *a*	Homologous and heterologous. Donors: rats and rabbits. Recipients: rats with aseptic peritonitis	P^{32}-phosphate *in vivo*	Determination of P^{32}-DNA in G-M counter	24 hours. Homologous cells in spleen and liver and heterologous cells in liver. More homologous cells in spleen and fewer in liver as compared to recipients without peritonitis. Fewer heterologous cells in liver as compared to recipients without peritonitis
Fichtelius 1957 *b*	Homologous and heterologous. Donors: rats and rabbits. Recipients: rats with aseptic peri-	P^{32}-phosphate *in vivo*	Determination of P^{32}-DNA in G-M counter	24 hours. Heterologous cells recovered as exudate cells.

Keohane & Metcalf 1958	Autologous, homologous and heterologous. Rats. Heterologous cells from guinea-pigs	Fluorescent dye *in vitro*	Fluorescence microscopy	During transfusion–2 hours. Lung, kidney, liver and spleen. Between $1\frac{1}{2}$ and 2 hours cells also in thoracic duct. No differences between autologous, homologous and heterologous cells
Fichtelius 1958 *a*	Homologous and heterologous. Rats. Heterologous cells from rabbits	P^{32}-phosphate *in vivo*	Determination of P^{32}-DNA in G-M counter	24 hours. Homologous cells in liver and spleen. Heterologous cells in liver
Fichtelius 1959 *a*	Heterologous. Donors: rabbits immunized and non-immunized with *S. typhi*. Recipients: rats immunized with *S. typhi*	P^{32}-phosphate *in vivo*	Determination of P^{32}-DNA in G-M counter	24 hours. Cells from immunized donors in liver and possibly also in epidermis. Cells from non-immunized donors in liver, epidermis and bone marrow. In liver fewer cells from immunized donors than from non-immunized donors
Fichtelius & Diderholm 1959 *b*	Homologous. Rats. Recipients immunized with *S. typhi*	P^{32}-phosphate *in vivo*	Determination of P^{32}-DNA in G-M counter	24 hours. Liver and spleen. Probably more cells in these organs as compared to non-immunized recipients
Fichtelius 1959 *b*	Homologous. Guinea-pigs. Donors sensitized and non-sensitized with dinitrochlorobenzene. Recipients sensitized with dinitrochlorobenzene	P^{32}-phosphate *in vivo*	Determination of P^{32}-DNA in G-M counter	24 hours. Spleen and liver. In these organs fewer cells from sensitized donors than from non-sensitized donors
Diderholm & Fichtelius 1959 *a*	Homologous. Guinea-pigs	P^{32}-phosphate *in vivo*	Apposition autoradiography	48 hours. Spleen (red pulp) and hepatic lymph node (non-follicular regions)

TABLE 10. *Review of experiments in which lymphocyte suspensions made of lymph nodes were transfused.*

Author	Type of transfusion. Experimental animals	Method of labelling the lymphocytes	Method of identifying the lymphocytes after transfusion	Interval after transfusion. Location of the lymphocytes
Osogoe 1950	Homologous. Rabbits	No label	Examination of histological sections	Shortly after transfusion. Lung.
				48-72 hours. Liver (interlobular spaces) and spleen (perifollicular regions)
Farr 1951	Autologous and heterologous. Rabbits. Heterologous cells from guinea-pigs	Fluorescent dye *in vitro*	Fluorescence microscopy	2–3 and 12 hours. Autologous cells in bone marrow, thymus (medulla and cortex), mesenteric lymph node (medulla and cortex), Peyer's patches and spleen (white pulp). After 12 hours cells in bone marrow had turned into myelocytes. No heterologous cells recovered
Fichtelius 1958 *a*	Homologous and heterologous. Rats. Heterologous cells from rabbits	P^{32}-phosphate *in vivo*	Determination of P^{32}-DNA in G-M counter	24 hours. Homologous cells in liver, bone marrow and probably also in lymph nodes and lung. Heterologous cells in liver
Fichtelius 1959 *a*	Heterologous. Donors: rabbits immunized and non-immunized with *S. typhi*. Recipients: rats immunized with *S. typhi*	P^{32}-phosphate *in vivo*	Determination of P^{32}-DNA in G-M counter	24 hours. No cells recovered from immunized donors. Cells from non-immunized donors in liver and lung
Diderholm & *Fichtelius* 1959 *b*	Homologous. Guinea-pigs	P^{32}-phosphate *in vivo*	Apposition autoradiography	48 hours. Spleen (red pulp), hepatic and mesenteric lymph nodes (non-follicular regions)

Author	Site from which the lymphocytes were taken. Type of transfusion. Experimental animals	Method of labelling the lymphocytes	Method of identifying the lymphocytes after transfusion	Interval after transfusion. Location of the lymphocytes
Weisberger et al. 1951	Intestinal lymphatic vessel. Homologous. Rats	P^{32}-phosphate *in vivo*	Determination of P^{32} in G-M counter	10 minutes. Lung, spleen and possibly also kidney and liver
Gowans 1959 *a*	Thoracic duct. Isologous. Rats	P^{32}-phosphate *in vivo*	Determination of P^{32} in G-M counter	12 hours. Cervical, mesenteric and submaxillary lymph nodes, thoracic duct and spleen
Ambrus & Ambrus 1959	Thoracic duct. Homologous. Dogs	Fluorescent dye *in vitro*. P^{32}-phosphate *in vivo*	Fluorescence microscopy. Determination of P^{32}-DNA in G-M counter	50–150 minutes. Cells in Thiry-Vella intestinal loops
Shorter & Bollman 1960	Intestinal lymphatic vessel. Autologous and homologous. Rats	Cr^{51}-chromate and P^{32}-phosphate *in vitro* and *in vivo*	Determination of Cr^{51} in scintillation counter and of P^{32} in proportional counter	$\frac{1}{2}$, 4, 16, 24, 48, 66 and 72 hours. Spleen, bone marrow, thymus, mesenteric lymph node, liver and small intestine. No differences between autologous and homologous cells
Everett et al. 1960 (Preliminary report)	Thoracic duct and blood. Homologous. Rats	H^3-thymidine *in vivo*	Autoradiography (coating with melted emulsion)	4 and 18 hours. Cells from thoracic duct in thoracic duct. 6, 12, 24, 36 and 48 hours. Cells from blood in thoracic duct, spleen, mesenteric and cervical lymph nodes, bone marrow and Peyer's patches

1958 *a*), homologous and heterologous lymphocytes are differently distributed after transfusion. In *Farr's* (1951) work only autologous lymphocytes could be traced, while no heterologous ones were recovered at all. *Keohane & Metcalf* (1958) noted no differences between autologous, homologous and heterologous lymphocytes, but this may be because the cells could not be traced later than 2 hours after the transfusion. *Fichtelius* (1953 and 1960) suggested that the difference in behaviour between homologous and heterologous transfused lymphocytes was due to different immunological states of the donors. The homologous cells derive from donors with an acquired immunity to more or less the same infections as the recipients, while in the case of heterologous transfusions the donors have been exposed to other antigens.

This suggestion is supported by the fact that immunization of donors influences the destination of transfused cells. *Fichtelius* (1959 *a*) found that fewer lymphocytes migrated to the liver of the recipients when the donors were immunized with *S. typhi* than when the cells were taken from non-immunized animals. The same author (*Fichtelius* 1959 *b*) also showed that fewer lymphocytes from donors sensitized to dinitrochlorobenzene were recovered in the spleen and liver compared with lymphocytes from non-sensitized donors. In the investigation described in Chapter IV a larger proportion of the lymphocytes recovered in the spleen was localized in the white pulp than was the case with the H^3-labelled lymphocytes in non-immunized animals (Chapter II). It is, however, difficult to establish how far this was due to immunization, since the times at which the animals were killed differed in the investigations.

In many of the investigations listed in the Tables it has been impossible to study the location of the lymphocytes within the organs. Exceptions are the investigations with unlabelled cells, those using autoradiography and those where the cells had been labelled with fluorescent dye. *Osogoe* and co-workers (1950, 1950 and 1954) recovered transfused thymus as well as lymph node lymphocytes in the perifollicular regions of the spleen. *Farr* (1951) located the transfused lymph node lymphocytes in the spleen to the white pulp, while in the experiments with apposition autoradiography thymus and lymph node cells were traced to the red splenic pulp (*Diderholm & Fichtelius* 1959 *a* and *b*). It is difficult to compare the observations by *Osogoe* and co-workers, with the present results,

since the number of cells transfused by the former workers was very large and the size of the spleen increased considerably. Concerning the observations by means of apposition autoradiography it appears very likely that cells were present in the white pulp as well, but that they were too few to be registered. However, the present results do not agree with *Farr's* observation concerning the distribution of cells within the spleen. To what extent this may be due to the facts that different types of lymphocytes and different kinds of animals were used or that the times between the transfusion and killing the animals were different it is impossible to say.

In the liver the lymphocytes were accumulated in the interlobular spaces, according to *Osogoe* and co-workers (1950, 1950 and 1954). In the present investigations the cells were recovered principally within the lobules. Here, too, it is impossible to say what this difference is due to.

In the lymph nodes the lymphocytes were recovered in the cortex as well as the medulla both in *Farr's* (1951) investigation and in the investigations with apposition and stripping film autoradiography. No cells could be traced to the follicles by means of apposition autoradiography, but this can be explained by the low efficiency of the film. In *Farr's* work the location of the cells in the cortex is not described in detail.

The investigations described in Chapters II, III and IV revealed nothing to indicate that the transfused thymus lymphocytes could be transformed into other types of cells. As previously mentioned, *Farr* (1951) observed labelled myelocytes that he maintained had been formed by a transformation of the transfused lymphocytes, but this assertion was criticized by *Keohane & Metcalf* (1958). *Fichtelius* (1957 *b*) has shown that transfused thymus lymphocytes can turn into exudate cells. No transformation of lymphocytes into other types of cells has been observed in any of the other investigations listed in the Tables, but *Bond, Fliedner, Cronkite, Rubini, Brecher & Schork* (1959) have shown in preliminary studies on parabiotic rats that when H^3-thymidine has been administered to an animal H^3-labelled cells in the blood of that animal probably migrate to the other animal where they are transformed into undifferentiated cells. These may then form the stem cells for other types of cells.

In the investigations described in Chapters II and III no labelled

cells were observed in the blood. This may very well agree with the results obtained by *Farr* (1951) and *Keohane & Metcalf* (1958). *Farr* states that most of the transfused lymphocytes were removed from the blood during the first 90 minutes and after 4 hours only stray cells were observed. *Keohane & Metcalf* recovered only a few transfused lymphocytes in the blood after 1 hour.

To sum up we may say that as a rule the results of the present investigations broadly agree with the results of the other investigations. Interesting exceptions are the observations concerning a migration of lymphocytes to the intestinal wall and skin. Concerning the location of the transfused lymphocytes within the organs and the ability of these cells to turn into other types of cells the observations made in the present investigations also usually agree with those in others.

Interpretations of results with regard to the destination and functions of lymphocytes after leaving the blood

The transfusion investigations described in Chapters II and III showed that the thymus lymphocytes accumulated principally in the spleen and liver and to a lesser extent in the mesenteric lymph node, lung and bone marrow. In the investigation in Chapter IV the transfused cells were traced to the spleen, very few being observed in the mesenteric lymph node. Even though these investigations suffer from certain weaknesses they do give some information concerning the migration of the cells. The results do not lend themselves to an exact interpretation of the extent of the lymphocyte migration to the different organs under physiological conditions, but they indicate very strongly that more thymus lymphocytes migrate to the spleen and liver than to the mesenteric lymph nodes, lungs and bone marrow. Further, the experiments indicate that few or no thymus lymphocytes migrate to the skin and the walls of the small intestine.

The investigation where subtotal thymectomy was carried out on immunized and non-immunized animals gave no information concerning the extent of cell migration from the thymus to the spleen and lymph nodes. The results of the investigation into the number and distribution of labelled cells in the spleen and thymus at different intervals after the administration of H^3-thymidine may,

however, very well agree with the conclusion of the transfusion investigations that relatively many lymphocytes accumulate in the spleen.

Neither the transfusion investigations nor the investigation with subtotal thymectomy gave any evidence of a transformation of thymus lymphocytes into other types of cells.

In the Introduction we briefly discussed the destination and function of lymphocytes after leaving the blood. The following theories were put forward. A recirculation of the cells to the blood via lymph nodes and lymphatic vessels, a migration of the cells through the epithelium of the digestive and respiratory tracts, a migration to the epidermis followed by a transformation into epidermal cells, a migration to the bone marrow followed by a transformation into myelopoietic and erythropoietic cells, a migration to the liver and spleen and a migration to the connective tissue all over the body. In the spleen the lymphocytes could function as stem cells for the plasma cells.

The results obtained in the investigations support only some of these theories. The presence of a few transfused lymphocytes in the mesenteric lymph node may indicate a recirculation via the lymph nodes but does not support the assumption that recirculation takes place on a larger scale. No transfused cells were observed in the walls of the small intestine or the skin. There was thus nothing to indicate an excretion of the cells through the intestinal wall or a transformation of the lymphocytes into epidermal cells. How far the transfused lymphocytes in the lung are eliminated from the body via the alveolar walls it is impossible to say, but it seems more probable that surplus lymphocytes accumulate in the lung, whence they are distributed to other parts of the body. Labelled lymphocytes were recovered in the bone marrow, while no labelled cells belonging to the myelopoietic or erythropoietic series could be observed. Thus the results do not support the theory that lymphocytes can function as stem cells for other blood cells. A rather large part of the transfused cells was traced to the spleen and liver. Though those in the spleen occurred in regions rich in plasma cells, there were no indications of a transformation of lymphocytes into plasma cells. It is not possible to generalize concerning the distribution of the cells in connective tissue sites, since the interlobular spaces of the liver were the only such sites, which regularly showed transfused cells. It must be emphasized that these

results mainly refer to thymus lymphocytes. Had other kinds been used or if the organs had been examined either sooner or later after the transfusion, it is possible that more support would have been gained for the above-mentioned theories about the destination and function of lymphocytes.

In the Introduction it was pointed out that the lymphocytes may have a function in nourishing other cells by producing and transporting nucleoproteins, which after their death are utilized by these cells. It has been supposed that this function is especially pronounced in organs having active cell proliferation (*Andreasen* 1959). The accumulation of the transfused lymphocytes in the spleen, lymph node and bone marrow may be connected with the possibility that the lymphocytes may act as trephocytes. In the spleen and lymph nodes it is possible that the nucleoproteins are largely used for the formation of the lymphocytes themselves. This question is of great significance in connection with determinations of the life-span of the lymphocytes by means of the incorporation of radioactive isotopes in the DNA of the cells (*Osgood et al.* 1952, *Ottesen* 1954 and *Hamilton* 1956, 1957 and 1959). The results obtained indicate that lymphocytes have a mean life-span of several weeks. However, as suggested by *Hamilton*, the labelled DNA of dead lymphocytes may be utilized by other lymphocytes thus invalidating the life-span values. It is also very difficult to state anything definite concerning the function of the lymphocytes in the liver. In this organ, too, it has been supposed that lymphocytes can function as trephocytes (*Kelsall & Crabb* 1958), but here as in the other organs these cells most probably have other functions as well.

CHAPTER VIII

General Summary

In the Introduction (Chapter I) a survey is made of the literature concerning the production of lymphocytes, their release from the producer organs, the length of their stay in the blood, their fate after leaving the blood and their function. Among the topics discussed is the extent to which lymphocytes from the thymus differ from lymphocytes formed in other tissues. It appears that current knowledge is limited concerning the destination of the lymphocytes after leaving the blood, in what organs, and particularly where in these, they accumulate and their function there. These problems have largely been approached by means of transfusing labelled lymphocytes. The methods so far used have certain drawbacks, however. Either it has been impossible to make observations at the cellular level, or the labelling methods have not permitted sufficiently long experimental intervals.

The aim of the present investigations has been to study to what organs and regions of these lymphocytes, especially thymus lymphocytes, migrate and their ability to turn into other types of cells in immunized and non-immunized animals. In transfusion experiments lymphocytes were labelled with radioactive isotopes and registered by means of stripping film autoradiography, a technique eliminating the drawbacks inherent in the methods previously used. The fate of the lymphocytes was further studied by means of an investigation into the effect of subtotal thymectomy upon the spleen and lymph nodes, and, finally, by an investigation into the number and distribution of labelled cells in the spleen and thymus at different intervals after the administration of H^3-thymidine.

Autologous transfusions of thymus lymphocytes labelled *in vitro* with H^3-thymidine or S^{35}-methionine were made on guinea-pigs (Chapters II and III). Autoradiograms of the thymus cell suspensions showed a considerably larger number of lymphocytes labelled with S^{35}-methionine than with H^3-thymidine, which presumably was

incorporated mainly in the cells synthesizing deoxyribonucleic acid. Thus about 10% of the lymphocytes were labelled with H^3-thymidine, while about 45% were labelled with S^{35}-methionine. Most of the H^3-labelled lymphocytes were medium-sized, whereas the majority of S^{35}-labelled lymphocytes were small. The animals were killed 8 hours after transfusion, and the spleen, liver, mesenteric lymph node, lung, intestine, skin, blood and bone marrow examined by means of stripping film autoradiography. Labelled lymphocytes were found mainly in the spleen and liver and to a lesser extent in the mesenteric lymph node, lung and bone marrow, while no labelled cells were observed in the intestinal wall, skin or blood. In the spleen about 90% of the labelled lymphocytes were situated in the red pulp, with a tendency to accumulate in the regions bordering on the white pulp. Most of the lymphocytes traced to the liver were present in the lobules, and in the lung the cells were recovered in the alveolar walls. In the mesenteric lymph node the labelled cells were diffusely spread over both medulla and cortex with the exception of the follicles, in which only stray cells were found. As a rule autoradiograms of the organs revealed more labelled lymphocytes after transfusion of S^{35}-labelled cells than after H^3-labelled cells. This was to be expected, seeing that, according to the autoradiograms of the thymus cell suspensions, the number of transfused labelled lymphocytes was larger with S^{35}-methionine than when H^3-thymidine was used. The distribution of the H^3- and S^{35}-labelled lymphocytes among and within the organs was more or less the same, however. No support was obtained for the theory that lymphocytes can turn into other types of cells. That the overwhelming majority of labelled lymphocytes observed almost certainly were those transfused or lymphocytes formed by mitosis of the transfused cells was established by transfusions of lymphocytes killed after labelling and also by comparisons between the degrees of labelling of the lymphocytes.

The behaviour of the lymphocytes and particularly their capacity to turn into plasma cells was studied in immunized guinea-pigs by means of autologous transfusions of thymus lymphocytes labelled *in vitro* with H^3-thymidine (Chapter IV). The transfusions took place under secondary response to *S. typhi*, and the animals were killed after $3\,^1/_3$ and $5\,^1/_3$ days. The spleen and mesenteric lymph node were examined with stripping film autoradiography. Nothing

was found to indicate that lymphocytes are able to change into plasma cells or other types of cells. Labelled lymphocytes were mainly observed in the spleen, while the lymph node contained only a few cells. About 65% of the labelled lymphocytes in the spleen were situated in the red pulp, i.e. a smaller proportion than in non-immunized animals (Chapters II and III). In the lymph node they were diffusely spread over both medulla and cortex with the exception of the follicles, in which only stray cells were occasionally found. By means of transfusions of lymphocytes killed after labelling it was shown that practically all the labelled lymphocytes observed were very probably those transfused or had been formed by mitosis of the transfused cells.

In order to approach the problems of lymphocyte migration and transformation in another way, a study was made of the influence of subtotal thymectomy on the weights of spleen and lymph nodes, on the volumes of the red and white splenic pulps and on the plasma cell content of the spleen (Chapter V). Non-immunized and *S. typhi*-immunized guinea-pigs were used. The results of this investigation were inconclusive concerning lymphocyte migration and transformation, however.

As mentioned above, the transfusion experiments with non-immunized animals showed that the thymus lymphocytes had a strong tendency to accumulate in the red pulp of the spleen (Chapters II and III). Similar results were obtained in a previous investigation with transfused lymph node lymphocytes: the number of transfused cells per field of vision was greater in the red pulp than in the white. In order to obtain indications that this agrees with the behaviour of lymphocytes under physiological conditions, the spleen and thymus of rats were examined by means of stripping film autoradiography 1 and 6 hours as well as 4 days after the administration of H^3-thymidine (Chapter VI). After 1 hour the number of labelled cells per field of vision was greater in the red splenic pulp than in the white. Another 5 hours later the number of labelled cells in the red pulp had increased considerably, while no great difference was observed concerning the number of labelled cells in the white pulp. The autoradiograms of the spleens of animals killed after 4 days gave an entirely different picture: the white pulp contained more labelled cells per field of vision than the red. The differences be-

tween the labelling of the two pulps at the different intervals may be explained by a migration of lymphocytes to the spleen, where, as suggested by the transfusion experiments, they have a greater tendency to accumulate in the red pulp rather than in the white. The results may be explained in other ways, too, and some of these as well as the behaviour of the thymus lymphocytes during the course of the experiment are discussed.

In the General Discussion (Chapter VII) it was asked to what extent transfusion experiments with lymphocytes can give information regarding the migration of the lymphocytes under physiological conditions. The choice of thymus lymphocytes for transfusion and of H^3-thymidine and S^{35}-methionine for labelling is justified. The radiosensitivity of lymphocytes and the possibility that factors other than the ionizing effect of H^3 and S^{35} have affected the autoradiographic film are discussed. In the part of the General Discussion dealing with the results of the present investigations a comparison is made with the results of earlier research where labelled or unlabelled cells have been transfused and identified with other methods. Further, the results are examined with regard to some theories about the destination and functions of lymphocytes after their disappearance from the blood.

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